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Specification and Drawings, as originally filed, with Application for Patent Serial No:
2,436,104, on July 14, 2003, by **MCGILL UNIVERSITY**, assignee of Nahum Sonenberg
and Marcelo Lopez-Lastra, for "Method for Inducing Hepatitis C Virus (HCV) Replication
In Vitro, Cells and Cell Lines Enabling Robust HCV Replication and Kit Therefor".

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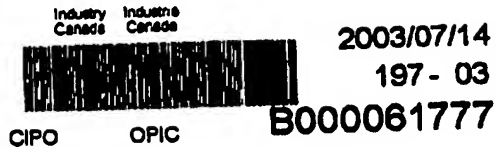
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(CIPD 68)
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PETITION FOR GRANT OF A PATENT



1. The applicant MCGILL UNIVERSITY

whose complete address is 845 Sherbrooke St. W., Montreal, Quebec, Canada H3A 2T5

requests the grant of a patent for an invention, entitled "**METHOD FOR INDUCING HEPATITIS C VIRUS (HCV) REPLICATION *IN VITRO*, CELLS AND CELL LINES ENABLING ROBUST HCV REPLICATION AND KIT THEREFOR**"

which is described and claimed in the accompanying specification.

2. ~~This application is a division of application number filed in Canada on.~~

3. ~~(1) The applicant is the sole inventor.~~

(2) The inventors 1) SONENBERG, Nahum; and 2) LOPEZ-LASTRA, Marcelo

whose complete addresses are 1) 4609 Blossom Avenue, Côte St-Luc, Quebec, Canada H4W 2S9; and 2) 6201 Côte St-Luc Road, Apartment 33, Hampstead, Quebec, Canada H3X 2H2

and the applicant own in Canada the whole interest in the invention.

4. The applicant requests priority in respect of the application on the basis of the following previously regularly filed application:

Country of filing	Application number	Filing date
N/a	n/a	n/a

ABSTRACT OF THE DISCLOSURE

The present invention relates to hepatitis C virus (HCV). More particularly, the invention relates to the development of a
5 tool suitable for the search and validation of novel HCV antiviral drugs and therapies (e.g. vaccine). The invention further relates to methods for inducing HCV replication *in vitro*, and more particularly to a simple *in vitro* replication assay of HCV which enables productive and sustained infectious HCV production.

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TITLE OF THE INVENTION

METHOD FOR INDUCING HEPATITIS C VIRUS (HCV) REPLICATION
IN VITRO, CELLS AND CELL LINES ENABLING ROBUST HCV
REPLICATION AND KIT THEREFOR

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FIELD OF THE INVENTION

The present invention relates to hepatitis C virus
(HCV). More particularly, the invention relates to the development of a
tool suitable for the search and validation of novel HCV antiviral drugs
and therapies (e.g. vaccine). The invention further relates to methods
for inducing HCV replication *in vitro*, and more particularly to a simple *in*
vitro replication assay of HCV.

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BACKGROUND OF THE INVENTION

The hepatitis C virus (HCV) is an enveloped RNA virus
of the *Flaviviridae*, which is classified within the Hepacivirus genus.
HCV is an important etiologic agent of chronic liver diseases. At this
time HCV infection is one of the primary causes of liver transplantation
in the US and other countries. Acute infections are usually subclinical or
associated with mild symptoms, but the virus persists in more than 80%
of infected individuals despite evidence of active, antiviral
immunological response (J. Viral Hepatitis 1997, 4:31-41; Hepatol 1998,
28:939-944; J. Viral Hepatitis 1999, 6:36-40). It is estimated that more
than 170,000,000 people are seropositive world-wide (Hepatology
1997, 26:62S-65S). The long-term outcome of HCV persistent infections
are varied, and they can range from an apparently healthy carrier state

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to chronic active hepatitis, liver cirrhosis, and eventually hepatocellular carcinoma (N Engl J Med 1992, 327:1899-1905; Hepatology 1990, 12:671-675). The mechanism of such pervasive persistence is entirely unknown. To date, there is no vaccine for HCV and the only available
5 therapy for chronic viral infections is treatment with Interferon alpha (IFN- α) either alone or in combination with the nucleoside analogue ribavirin (J Hepatol 1999, 30:956-961; Mol Immunol 2001, 38:475-484). Unfortunately, only ~40% of treated patients develop a sustained response that is defined by absence of viral RNA for more than 6
10 months after cessation of therapy (J. Viral Hep. 1999, 6:35-47). Moreover, during IFN- α treatment selection of viral variants resistant to INF- α occurs frequently (Microbes & Infection 200, 2:1743-1756). In addition, ribavirin can be used to treat patients. HCV resistance to ribavirin is also common. The search for HCV drugs as well as the
15 development of an HCV vaccine is severely hampered by the lack of an efficient tissue culture or simple animal system for the study of replication and HCV pathogenicity. The only animal models currently available for the study of this virus are the chimpanzee and a mouse which possesses a chimeric human liver (Antiviral Research 2001, 52:1-
20 17; Nat Med 2001, 7:927-933). These facts cast HCV as an emerging human pathogen of extreme medical significance (J Viral Hepat 1999, 6:35-47).

There thus remains a need to provide a simple assay for HCV replication which would enable the study of HCV replication
25 and/or pathogenesis and enable the development of a treatment or prophylaxy for HCV infections. There also remains a need to provide a

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HCV replication system which enables the screening of anti-HCV compounds which can act in a larger number of stages of the HCV life cycle such as infection, replication, translation and assembly. There also remains a need to provide a system which enables the replication
5 of HCV from a patient so as to enable simpler and more efficient genotyping thereof and/or phenotyping (e.g. to identify its resistance/sensitivity characteristics toward anti-viral compounds).

While HCV infects a large number of individuals, no efficient treatment or vaccine has been developed, despite a significant
10 effort by the pharmaceutical industry. Thus, most companies with existing programs in the anti-infective area are focused towards the discovery of agents that are active against this virus. Thus far the human immunodeficiency virus (HIV) has provided a useful strategy for HCV antiviral drug development (Drug Discov Today 1999, 4:518-529).
15 In fact the understanding of the function of anti-HIV drugs has outlined the research platform of most of the companies screening for anti-HCV drugs. Both viruses share interesting features. They lead to chronic infection, are highly mutable, and they code for specific enzymes that are not expected to be present in a normal non infected cell. Based on
20 the results of HIV therapy, it is possible that a combination therapy involving at least two drugs directed against separate targets will be more effective at reducing HCV load and preventing the emergence of resistant strains than monotherapy. As the selected targets against HIV
25 have been the viral encoded protease and the viral reverse transcriptase, it is not surprising to find that HCV protease and RNA dependent RNA polymerase have often been mentioned as candidate

antiviral targets. As judged by the lack of disclosures, the discovery of anti-HCV agents has not been successful despite the functional similarity of several HCV-enzymes with known targets from other antiviral programs. Admittedly, part of this failure is because of the lack
5 of a tissue culture system, which in turn limits primary screens to isolate viral protein targets. Interestingly, despite the fact that the enzyme assays to test HCV protease are known, the discovery of a potential drug candidate has met with little success. Taken together, it might be
10 concluded that putative chemotypes for inhibition of HCV-targets are poorly represented in most industrial compound collections (Drug Discov Today, 1999, 4:518-529).

Should a series of novel anti-HCV drugs be developed, to advance these agents into the drug development pipeline, several issues will need to be addressed, notably, their
15 mechanism of action. Unfortunately, tissue culture and *in vivo* control experiments using whole virus are required to better determine the mode of inhibition. As stated above, an efficient cell culture system for the replication of HCV has not yet been provided (Drug Discov Today 1999, 4:518-529; Antiviral Res 2001, 52:1-17; J Mol Biol 2001, 313:451-
20 464; Virus Res 2002, 82:35-32).

Attempts have been made, based on the use of human cells of hepatocytic and lymphocytic origin, but low and variable levels of replication and virus-induced cytotoxicity posed important problems. Primary hepatocytes (derived from a human donor) can be
25 infected with HCV isolated from serum of viremic patients, and the virus can be detected in the supernatant for several weeks after infection.

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HCV replication has been demonstrated by detection of minus-strand RNA, an intermediate of virus replication, in primary hepatocytes derived from a HCV-negative donor after infection with sera from HCV-positive patients. However, the availability of primary hepatocytes is limited, and their isolation is time-consuming and labor-intensive. Consequently, such tissue culture systems are generally considered unsuitable for intensive large-scale antiviral studies.

Another example of progress in this domain has been the construction of subgenomic selective replicons cloned from a full-length HCV consensus genome from an infected liver (Antiviral Res 2001, 52:1-17; J. Mol Biol 2001, 313:451-64; Virus Res 2002, 82:25-32). Following transfection in human hepatoma cells, these RNAs were found to replicate to high levels, allowing detailed molecular studies of HCV and testing of antiviral drugs. One drawback of this system, however, is that it only expresses the non-structural viral proteins (Science 1999, 285:110-3). Therefore, studies aimed at assessing target viral assembly and trafficking through the cytoplasm cannot be carried out, with this reconstituted viral system. In other words, such artificial system is of a more limited potential to identify antiviral agents.

As previously mentioned animal models currently exist to study HCV replication. Although the chimpanzee model has contributed significantly to the understanding of HCV infection, the high cost and availability of these animals limit the extent to which antiviral-drug or therapy studies can be carried out. Small laboratory animals, including mice, are not susceptible to infection with HCV. An alternative model such as a mouse model with a chimeric human liver has been

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generated (Nat Med 2001, 7:927-933). This system is considered laborious and is known to require special expertise to isolate and transplant human hepatocytes and maintain a colony of fragile immunodeficient mice with an approximately 35% mortality in newborns
5 due to a defect in blood coagulation (Nat Med 2001, 7:927-933). Nevertheless, when all the required conditions are met this mouse model can provide an interesting system for testing antiviral agents.

There thus remains a need to provide a simple *in vitro* system, which is suitable for the replication of HCV.

10 There also remains a need to provide an *in vitro* tissue culture system for the replication of complete HCV.

There further remains a need to provide a tissue culture system for HCV which enables the screening and development of drugs and therapies for essentially all the different stages of virus
15 replication such as virus entry, cytoplasmic replication [viral (-) and (+) strand synthesis], viral protein synthesis, virus assembly, virus trafficking, and virus release.

The present invention seeks to meet these and other needs.

20 The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

25 The invention relates to a simple *in vitro* culture system, which is suitable for the replication of hepatitis C virus (HCV).

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The invention further relates to an *in vitro* tissue culture system which enables the replication of complete HCV.

In addition, the invention relates to a tissue culture system for HCV which enables the screening and development of drugs and therapies for essentially all the different stages of virus replication such as virus entry, cytoplasmic replication [viral (-) and (+) strand synthesis], viral protein synthesis, virus assembly, virus trafficking, and virus release.

The present invention also provides the means to diagnose HCV. In addition, it enables an identification of the response of a particular strain of HCV, from a particular patient, to a candidate antiviral compound or to a known antiviral compound.

The present invention further relates to a method of activating the replication of HCV in PBMCs comprising obtention of same from a HCV-infection patient and activating the replication of HCV by incubating the PBMCs with an activation-inducing amount of at least one mitogen (e.g. activator).

The invention in addition relates to a co-culturing system for replicating HCV *in vitro* which comprises co-culturing PBMCs (or PBLs) infected with HCV, wherein the PBMCs have been activated and in which the HCV can replicate, together with a cell line, wherein the co-culturing enables infection of the cell line and replication of the HCV thereinto. In a particular embodiment of the present invention, the cell line is an immortalized cell line.

It is believed that the Applicant is the first to provide an *in vitro* cell system which enables replication of a native HCV.

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It is believed that prior to the present invention, while HCV could infect PBMCs, it was unknown that it could actively replicate in them. The present invention demonstrates HCV tropism for PBMCs and more particularly for PBLs. As known in the art, PBMCs are a mixture of cells which also include macrophages and PBLs (which can be obtained from PBMCs) contain about 85% T cells and 5% B cells.

It is also believed that this is the first demonstration that the HCV produced in an *in vitro* system is infectious and that sustainable replication of HCV can be achieved.

Before the present invention, large-scale production of HCV was unthinkable. The methods and *in vitro* system of the present invention enables active replication of HCV in cells for at least 9 days and opens the way to large scale production.

Prior to the present invention, no tissue culture technology currently existed to replicate HCV. The only animal models currently available for the study of this virus are the chimpanzee and mice models (mice with chimeric human livers). These animal based-system are laborious and require special expertise and facilities.

RNA interference can be used in accordance with the present invention using, for example, the teachings of 6,506,559.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and in which:

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Figure 1 shows the hepatitis C virus (HCV) genome organization;

Figure 2 shows the hypothetical model of the HCV replication cycle;

5 Figure 3 shows an experimental protocol. All experiments were performed with 1,000,000 cells/ml. T1 = anti-CD3 (1 μ g/ μ l final), IL-2 (final = 200 U). T2 = PHA (3 μ g/ μ l), IL-2. T3 = PHA, IL-2, SAC (1/10⁴). T4 = PHA, IL-2, SAC, IL-4 (final = 200 U);

10 Figure 4 shows PBMC and PBLC purification from blood samples;

Figure 5 shows the detection of HCV NS3 and NS5 proteins in cell extracts from treated PBMC from a HCV (+) patient;

15 Figure 6 shows a validation that the antibody used is decorating the NS3 translated (if positive) in the replicon system and that of the present invention activated (A) or non-activated (NA);

Figure 7 shows the time course of HCV-NS3 detection: PBMCs from patient MLL-001;

Figure 8 shows the time course of HCV-NS3 detection: PBMCs from patient MLL-002;

20 Figure 9 shows the detection of HCV-NS3 protein in treated (N3) PBMCs from HCV9+ donors;

Figure 10 shows the detection of virus like particles by scanning electron microscopy;

25 Figure 11 shows the electron microscopy of activated PBLCs and detection of virus like particles;

Figure 12 shows a virus partial purification;

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Figure 13 shows the detection of HCV core protein in supernatant of treated PBMC from an HCV(+) patient;

Figure 14 shows RNA quantification I (virus copies/ng total RNA);

5 Figure 15 shows an infection assay; co-culture;

Figure 16 shows infection of MT-4 cells RNA quantification II (virus copies/ng total RNA);

Figure 17 shows co-culture of Huh-7 and HCV (-) PBMCs;

10 Figure 18 shows co-culture of Huh-7 and HCV (+) PBMCs (SB006);

Figure 19 shows PHA activation of PBMCs from patient SB004 (HCV is not in T cells);

15 Figure 20 shows the detection of HCV (E2) on Daudi cells upon co-cultivation with infected PBMCs (the control for Fig.). Of note, Daudi cells are a B cell line;

Figure 21 shows a comparison of different activation treatments (PBMCs from donor MLL-010). T1 = PHA + IL-2. T2 = SAC + IL-2. T3 = T1 + T2; and

20 Figure 22 shows viral RNA in cell supernatant (real time RT-PCR). T1, T2, T3 are the same as for the preceding figure. Of note, further addition of IL-4 to T3 further increased activation.

Fig. 23 shows that HCV (+) and (-) strand RNA is produced de novo in activated PBLs. A) HCV-RNA was detected in
25 PBLs from an HCV positive donor by a one step reverse transcription-polymerase-chain reaction (RT-PCR) followed by a nested PCR

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amplification using primers that targeted the highly conserved 5' untranslated region (on-line material and methods). Total RNA, from either activated (P) or non-activated (N) cells, were prepared at the indicated times. RNA from Huh7 cells stably expressing the HCV replicon (Huh-Rep) (47) was used as positive control. RNA extracted from PBLs from an HCV negative donor and yeast tRNA were used as negative controls. B) Kinetics of HCV-RNA synthesis. PBLs from two positive donors, MLL-038 (Δ) and MLL-039 (O), were stimulated by method P. RNA was extracted at the indicated time of culture and the level of HCV (-) strand RNA was determined using the Roche LightCycler system. RNA levels were normalized against GAPDH and are reported as a fold variation relative to the amount of (-) strand RNA in non-treated PBLs. C, D) Bromo-uridine incorporation into de novo synthesized RNA was detected in by immunofluorescence using an anti-bromodeoxyuridine antibody. C) HCV positive donor MLL-069. D) HCV negative donor.

Fig. 24 shows that HCV proteins are produced in activated PBLs. PBLs were stimulated using method P. Protein extracts were prepared following five days of activation. A) Extracts from either treated (P) or non-treated (N) PBLs, from donor SB-1 were run side by side with extracts from Huh-7 cells expressing the HCV replicon (Huh-Rep) (47). NS3 was detected using polyclonal antibody K135. B) Extracts from PBLs, either treated (P) or non-treated (N), from a HCV negative donor were run side by side with extracts from donor SB-6. NS3 was detected using monoclonal antibody 1G3D2. C) Extracts from Huh-7 cells and Huh-Rep, were run side by side with extracts, either

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treated (P) or non-treated (N), from an HCV negative and positive donor. NS5B was detected using monoclonal antibody 5B-3B1 (48) or 5B-10 (IFA). D) Extracts from either treated (P or A) or non-treated (N) PBLs from different HCV positive donors were run side by side with
5 extracts from an HCV negative donor, Huh-7 or Huh-Rep cells. NS3 was detected using monoclonal antibody 1G3D2. E) Kinetics of NS3 synthesis following PBLC stimulation by methods P, S and PS. Extracts were prepared on the indicated days and NS3 was detected using monoclonal antibody 1G3D2. F, G, H) Kinetics of NS3 accumulation in
10 donors MLL-001, MLL-002 and MLL-010 after stimulation using method P. Extracts were prepared on the indicated days. An extracts from non-treated cells was prepared either on day 3 (F and G) or on day 2 (H). NS3 was detected using anti-NS3 monoclonal antibody 1G3D2 (F and G) or with an NS3 rabbit antiserum-RB (H). Actin or a non-specific
15 band, LC, identified by antibody 1G3D2, were used as loading controls. I, J, K.) siRNA silencing of HCV RNA. Core-siRNA or a non-specific RNA sequence (nsRNA) were electroporated into PBLs three days after stimulation. Proteins and RNA were extracted 48 hr later. I) NS3 and NS5B were detected with NS3 rabbit antiserum-RB and 5B-3B1
20 monoclonal antibody (48), respectively. Actin was used as an internal control. J) RNA levels were quantified by real-time PCR (method I, materials and methods). Absolute copy number of the HCV (+) strand transcripts (Δ) and the amount of GAPDH (O) RNA are shown. K) HCV RNA amounts were normalized against GAPDH. The ratio of
25 HCV/GAPDH was determined for the nsRNA and assigned an arbitrary

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value of 100. The Core-siRNA HCV/GAPDH ratios are expressed relative to the negative control.

Fig 25 shows that HCV Core protein was detected by indirect immunofluorescence in day 3 stimulated (P) PBLs from MLL-059, using the RR8 polyclonal antibody. Stimulated PBLs from an HCV negative donor were used as a control.

Fig. 26 shows that HCV is released from activated HCV positive PBLs. A, B) Supernatant from stimulated PBLs (method P) was collected and sedimented through a 20% sucrose cushion. A) Sedimented proteins were resolved by SDS 15%-PAGE, transferred to a nitrocellulose membrane (overnight, 30V) and detected using MAB255P monoclonal anti-core antibody (Maine Biotechnology Services, Inc.). HCV (-) corresponds to the negative control. B) RNA was analyzed by nested RT-PCR. RNA from Huh-Rep was used as a positive control. RNA from yeast tRNA, Huh-7, and an HCV negative donor were used as negative controls. C) PBLs from donor SB-5 were stimulated using methods B, P, and PS. Five days following activation, the supernatant was collected and sedimented through a 20% sucrose cushion. The quantity of HCV RNA was determined by real-time RT-PCR on the ABI Prism 7700 Sequence Detection System. D) Following metabolic labeling (³⁵S Met/Cys) of PBLs from donor MLL-035, the supernatant was sedimented through a 20% sucrose cushion. The sediment was resuspended and analyzed by a flotation gradient. Collected fractions were resolved on a SDS-15% PAGE, transferred to a nitrocellulose membrane and exposed to a Kodak Biomax MR film. E) Fractions were concentrated and HCV E2 glycoprotein visualized by

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Western blotting using monoclonal anti-E2 1864 (450-470AA) antibody.

F) RNA was extracted from the gradient fractions of Fig 4E. and the absolute quantity of HCV RNA was determined by real time RT-PCR.

G) Fractions 1-4 (L) and 5-11 (H) from the flotation gradient were concentrated and pooled. Proteins were resolved on a SDS-15% PAGE. HCV E2 glycoprotein was detected using monoclonal antibody 1864 (450-470AA). Core protein was visualized using monoclonal anti-core 515S (20-40AA) antibody. H) Activated PBLs from donors MLL-059 and MLL-064 were metabolic labeled for 12h with 35S-Met/Cys or 32P-orthophosphate. Supernatants were sedimented through a 20% sucrose cushion. The sediments were resuspended and analyzed by a flotation gradient. The amount of incorporated radioactivity in each fraction of the gradients was determined in a Beckman LS 6500 scintillation counter.

Fig. 27 shows that virus released from activated HCV positive PBLs is infectious. A) Schematic representation of the co-culture chambers used in these experiments. B) MT-4 cells were co-cultured with either treated (P) or non-treated (N) MT-4 cells, PBLs from two HCV negative donors or PBLs from donors SB-2 or SB-7. Extracts were prepared following six days of co-culture. NS3 was detected using monoclonal anti-NS3 antibody 1G3D2. LC indicates a non-specific band used as a loading control.

Figure 28 shows Bromo-uridine incorporation into *de novo* synthesized RNA and detected by immunofluorescence using an anti-bromodeoxyuridine antibody in PBLs from donor MLL-065.

Figure 29 shows the HCV replication cycle.

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Figure 30 shows a protocol to detect HCV RNA in PBLs.

Figure 31 shows the detection of HCV protein by immunoprecipitation.

5 Figure 32 shows the detection of HCV protein by Western Blot.

Figure 33 shows immunofluorescence of HCV (-) Control Polyclonal-anti Core RR8 (M. Kohara).

10 Figure 34 shows immunofluorescence of MLL-059 Anti-Core RR8.

Figure 35 shows immunofluorescence of MLL-059 Anti-Core RR8.

15 Figure 36 shows immunofluorescence of MLL-059 Anti-Core RR8.

Figure 37 shows immuno-electronmicroscopy of HCV protein using an anti NS3 antibody.

Figure 38 shows electron microscopy of cells showing HCV viral particle assembly.

20 Figure 39 shows an embodiment of a scheme for virus partial purification.

Figure 40 shows density determination of HCV viral particles purified according to Fig. 39.

Figure 41 shows that PBMC generate two HCV subpopulations that can be partially purified by density gradient.

25 Figure 42 shows an embodiment of a protocol to assess infectivity of isolated HCV.

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Figure 43 shows EBV-transformed B-Cell lines express HCV proteins when stimulated.

Figure 44 shows HCV(-) PBLs are infected with HCV when co-cultured with stimulated HCV(+) B-cell lines.

5 Figure 45 shows mechanisms that could explain the HCV-activation results.

Figure 46 shows Crosslinking to the HCV IRES.

Figure 47 shows PBMCs Activation and HCV IRES Crosslinking pattern.

10 Figure 48 shows Crosslinking competition to the HCV IRES.

Figure 49 shows Crosslinking competition to the HCV IRES.

15 Figure 50 shows Crosslinking competition to the HCV IRES.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the accompanying drawings which is exemplary and should not be
20 interpreted as limiting the scope of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The existence of extrahepatic reservoirs of hepatitis C virus (HCV) replication remains controversial. Several groups have
25 described the presence of hepatitis C virus (HCV) genomic sequences (plus-strand) and replicative intermediate (minus-strand) in peripheral

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blood mononuclear cells (PBMC). The association of HCV RNA with peripheral blood leukocytes has been documented since 1992 (Proc Natl Acad Sci USA, 1992, 89:5477; J Virol. 1993, 67:1953; Hepatology 1996, 23:205; J Virol, 1997, 70:3325-9; J Virol 1996, 70:7219-23; 5 Antiviral Research 2001, 52:1-17). However, the specificity of the methods used in these studies has been questioned. More recent reports, which used an optimized negative strand-specific reverse-transcriptase polymerase chain reaction (RT-PCR) assay, detected negative-strand HCV only in PBMC taken from post-transplant or 10 human immunodeficiency virus (HIV)-coinfected HCV patients, and not in PBMC from typical patients with chronic HCV infection. Of note, a number of studies have also reported that human B and T cell lines are capable of supporting a productive infection. However, the data supporting viral production was only based on RNA detection (Proc. 15 Natl Acad Sci USA, 1992, 89:5477; J Virol 1993, 67:1953; Hepatology, 1996, 23:205; J Virol, 1996, 70:3325-9; J Virol 1996, 70:7219-23; Antiviral Research 2001, 52:1-17). The validity of these data have been questioned (Laskus et al. 1998, see below). Moreover, PBMC obtained from HCV negative donors were successfully infected using HCV- 20 positive sera, demonstrating that PBMCs are permissive for HCV replication *in vitro* (J Gen Virol 1995, 76:2485-2491). However, replication of the virus therein was really low. Of note, only RNA was detected. Thus, prior to the present invention, it remained unclear whether HCV could actively replicate to workable levels in PBMCs.

25 Using an immunodeficiency (SCID) mouse model that allow long-term survival of human hematopoietic cells Bronowicki et al.

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(1998) presented strong evidence for persistence of HCV RNA in PBMCs obtained from HCV positive donors (Hepatology 1998, 28:211-218). The susceptibility of PBMC to HCV infection has been corroborated by *in situ* hybridization techniques showing both positive and negative polarity RNA strands in circulating and/or bone marrow recruited mononuclear cells. Recent reports have established that HCV is in fact associated to B cells. Based on the model of Epstein-Barr virus another B-cell-tropic virus, that remains latent while the host cell is quiescent but is reactivated and enters a lytic replication phase once the host cell is activated (J Virol Methods, 1988, 21:223-227; Annu Rev Microbiol 2000, 54:19-48). Bolsvert et al (2001) examined the possibility that HCV could replicate in peripheral B cells, but under altered physiological conditions, such as immunosuppression or cellular activation. The authors could not detect HCV replication in enriched B cells obtained from HCV positive donors upon cell stimulation with CD40L.

Considering the observations of Laskus et al. (1998) showing the presence of active HCV replication in lymphoid tissue in patients coinfecting with HIV (not in non-HIV infected patients), suggesting that co-infection of HIV would be required in HCV cell-based assay, and those of Bolsvert et al (2001), it was hypothesized that HCV replication in peripheral blood leukocytes (PBML) requires cell activation (e.g. in the mixture of the T-and B-cell population).

Until now, all studies of HCV replication have concentrated on documenting the presence of the replicative intermediate (minus-strand) RNA. However, the validity of these reports

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has been criticized because the presence of viral proteins was not demonstrated. It stands to reason that in order for replication to occur, protein expression is required. Therefore, in order to sustain the observations relating to activated PBMCs, non-structural (NS) HCV proteins were chosen as an indicator of viral replication. The studies presented hereinbelow clearly demonstrated that PBMCs obtained from HCV seropositive donors are able to support at least one cycle of viral replication upon activation. For this a simple method that actively induces virus replication within the infected cell was developed.

10 Most circulating leukocytes are in a resting state, but remain responsive to mitogenic signal that can induce cell activation. Lymphocyte activation in response to extrinsic signals results in either progression through the cell cycle, or activation of proapoptotic pathway(s) (*Cell* 1991, 65:921-923; *Science* 1996, 274:1664-1672).
15 Lymphocyte activation correlates with a strong increase in translation rates and expression of translation initiation factors (*J Immunol.* 1998, 160: 3269-3273). As shown therein, the change in the cellular environment associated with immune activation could induce HCV protein synthesis and initiate a cascade of events leading to an
20 impaired cell cycle and an enhanced viral replication.

 The activation of PBMCs (or PBLCs) is achieved using at least one mitogenic (or activating agent). In one particular embodiment, the activating agent is a mixture of antigen-nonspecific T and/or B cell activators (Anti-CD3 antibody, phytohemagglutinin (PHA),
25 CD40L, Staphylococcus aureus crown I (SAC), IL2 and IL4). Of course, it will be realized that other T and B cell activating agents exist and are

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well-known in the art. Such agents could be used in the methods and culture systems of the present invention. In one particular embodiment, Ag-specific T and/or B cell activating agents could also be used. It will also be understood that the present invention provides assays which
5 can be used to identify further activating agents, mixtures thereof or other nutrients which can further activate the HCV-producing cells of the present invention and/or promote a longer survival thereof in culture.

HCV non-structural proteins (NS3 and NS5) were detected by Western blot analysis (data not shown). Virus-like particles
10 could be detected within the infected cells by electron microscopy demonstrating that viral proteins are assembling (data not shown). Viral particles could be isolated from the PBMCs supernatant. The presence of virus was evidenced from Western blot (anti-Core) analysis and genomic RNA detection by real time RT-PCR, this observation shows
15 that upon assembly viral particles were actively being liberated to the supernatant.

Moreover, using a co-culture method it was demonstrated that the HCV particles produced in PBMC could infect other cells. Non-limiting examples thereof include Huh-7 (liver), Daudi
20 (B-cell) and MT4 (T-cell) cell lines. Thus, not only can HCV replicate, and assemble in the tissue culture system of the present invention, it can also infect other cells. Infection was monitored by detection of viral RNA (real time RT-PCR). The results generated by these experiments will have a significant impact on the testing of anti-HCV agents. Of
25 course, it also serves as a proof of principle that PBMC are able to sustain HCV infection and generate infective HCV. Moreover these data

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strongly suggest that both the serum and PBMCs obtained from HCV positive donors can be used as a source of infectious virus to infect naïve cells such as monocyte and/or monocyte-derived dendritic cells (DCs). Therefore, the instant invention which enables the infection of cells with HCV is by itself a significant achievement.

A novel tool for developing a HCV vaccine

Adoptive transfer of donor-derived virus-specific T cells generated in cultures with antigen-bearing autologous monocyte-derived dendritic cells (DCs) has attracted considerable attention as a promising tool to generate a strong immune response (*Int. J. Cancer*. 2001, 94:459-73; *Exp. Hematol.* 2001, 29:1247-55; *Trends Mol. Med.* 2001, 7 :388-94). This technique has not only proved useful as an alternative anti-cancer strategy but also as a novel anti-virus therapy. For example, when DCs were pulsed with human cytomegalovirus virus (HCMV) antigen and cocultured with autologous peripheral blood lymphocytes from HCMV-seropositive individuals, there was an increase in the numbers of cytolytic T cells. This technique was used to enhance immunity in HCMV-seropositive transplant patients (*Blood*. 2000, 97: 994-1000).

Now having developed a technology to infect cells with HCV, it becomes possible to adapt the dendritic cells (DCs) technology mentioned above, to generate T-cell responses to HCV. Advantages for using DCs for this purpose include: i) they are considered the most potent of the antigen-presenting cells (APCs) (*Blood*. 1997, 90:3245-3287; *Nature*. 1998, 392:245-252); ii) their role in resistance against experimental malignancies and infections is well documented (*J.*

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Immunol. 1998, 161:2094-2098; *J. Virol.* 1998, 72:3812-3818); iii) DCs can be easily generated from bone marrow, cord blood, and peripheral blood; iv) DCs have the unique ability to process exogenously supplied antigen efficiently and present peptides on both class 1 and class 2 HLA molecules along with an array of costimulatory molecules (*Nature*. 1998, 392:245-252; *Nature*. 1999, 398:77-80). The presentation of both helper and CTL-defined epitopes suggests that both CD4+ and CD8+ HCV-specific T cells will be generated. This will allow both the generation of cytolytic effector function and the potential for re-establishment of longer-term immune memory, which may be important in preventing subsequent viral reactivation; vi) The lack of an absolute knowledge of the presented peptides means that this technique can be used for patients of any HLA type and will trigger T-cell reactivity to undefined immunogenic determinants, thereby allowing a greater potential for augmentation of a broader T-cell response. It is thus expected that this will reduce the possibility that selective pressure will be applied to HCV *in vivo*. Based on the foregoing, it is predicted that the approach described herein (together with possible adaptations by a person of ordinary skill using the knowledge in the art) will contribute significantly to the design of a vaccine therapy towards HCV infection.

The present invention is illustrated in further detail by the following non-limiting examples.

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EXAMPLE 1**Robust Hepatitis C virus replication in peripheral blood lymphocytes from infected donors**

There is considerable evidence that hepatitis C virus (HCV) resides in an extrahepatic reservoir. Although peripheral blood lymphocytes (PBLs) have been suspected of harboring HCV, virus production was not achieved in these cells despite many attempts. Here, we show that PBLs from HCV positive, injection drug users, harbor the virus and support viral replication. HCV replication was activated by *ex vivo* cell stimulation, with the use of a mixture of T and B cell activators. The presence of viral positive and negative RNA strands and HCV proteins is documented. Virus particles were isolated from cell supernatant and analyzed by density gradients centrifugation. Virus structural proteins and viral RNA could be readily detected in the supernatant of activated PBLs by Western blotting and real time RT-PCR, respectively. Virus particles contain *de novo* synthesized genomic RNA and structural proteins as shown by metabolic labeling with ³²P-orthophosphate and ³⁵S-labeled aminoacids. Finally, HCV particles, released from cells, are infectious as demonstrated by co-culturing. Studies using this novel HCV replication system should contribute to the understanding of the virus life cycle, host-virus relationship, pathogenesis and importantly to the discovery and validation of new anti-HCV agents.

Hepatitis C virus (HCV) is a significant etiologic agent of chronic liver disease (1). It is estimated that more than 170 million people world-wide are seropositive. About 85% of primary infections

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become chronic, and ~20% of patients with chronic HCV develop serious complications, such as liver cirrhosis, end-stage liver disease, hepatocellular carcinoma, and death due to liver failure (2). To date, there is no vaccine against HCV and the most effective therapy is treatment with peginterferon in combination with ribavirin (3, 4). The search and validation of novel HCV drugs is severely hampered by the lack of a robust cellular system that supports virus replication. These facts cast HCV as a human pathogen of extreme medical significance.

HCV is an enveloped RNA virus of the *Flaviviridae* family, classified within the Hepacivirus genus. It contains a 5'uncapped positive strand RNA genome of 9.4 kb, that possesses two overlapping open reading frames: one is translated into a single polyprotein of 3010 aminoacids, while the other yields a 17 kDa protein (5-7). The viral polyprotein is processed to generate at least 10 different structural and nonstructural proteins (5, 6). The genome of HCV is highly heterogeneous and the virus circulates as quasispecies in a single infected individual (8). HCV is primarily hepatotropic, but it has also been implicated in lymphoproliferative diseases such as mixed cryoglobulinaemia, B-cell non-Hodgkin's lymphoma, and Sjögren's syndrome (9). The case for HCV replication in PBLs is suggested by the following observations: a) PBLs from HCV positive donors are capable of transmitting viral infection when inoculated into chimpanzees (10), and b) HCV minus-strand RNA can be detected in PBLs from HCV carriers upon injection into SCID mice (11). However, despite the growing evidence that supports HCV entry into PBLs, viral RNA synthesis is still a matter of debate and virus replication in PBLs has not

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been demonstrated (9, 12). Detection of HCV genomic sequences (plus-strand) and replicative intermediates (minus-strand) in PBLs from chronically infected donors (13-16) or infected chimpanzees has been reported (17, 18). But, the presence of viral proteins or virus particles has never been documented. To examine HCV extrahepatic replication, we used PBLs from seventy-eight HCV positive, HIV-negative, injection drug users (IDUs; all obtained with written consent; table S1 detailing the available information on the participants is included in the on-line supplement). PBLs from the IDUs were treated with a mixture of T and B cell activators to show replication of HCV and infectivity of the de novo produced virus. The rationale behind the selection of IDUs as a source of PBLs is addressed below.

HCV (+) and (-) strand RNA and viral proteins are produced *de novo* in activated PBLs.

Viral RNA was detected in non-stimulated and stimulated PBLs from a HCV positive donor by nested RT-PCR (Fig. 23A). Viral RNA was not detected in HCV negative donors or in negative controls (Fig. 23A; Note that nested RT-PCR is neither strand specific nor quantitative). These results confirm early evidence showing that PBLs harbor HCV RNA (12-16). To obtain quantitative results, total RNA extracted from activated cells was subjected to a strand specific real time RT-PCR analysis to demonstrate the presence of HCV (-) RNA strand (Fig. 23B). The kinetics of HCV RNA induction was similar in activated PBLs from two carriers, MLL-038 and MLL-039 (Figs. 23B). The amount of (-) strand RNA increases slightly, but significantly, early (1 day) upon cell activation then decreases at later times (1-3 days), but

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increases again afterwards (5-7 days) (Fig. 23B). Although these kinetics are not readily explained, the presence of HCV (-) RNA strand supports the notion of virus replication in PBLs. HCV life cycle is cytoplasmic (5), therefore, to show that RNA synthesis occurs in the cytoplasm, bromo-substituted uridine (BrU) together with actinomycin D (ActD) was added to stimulated PBLs (19). Incorporated BrU was detected by immunofluorescence using antibodies to 5'-bromodeoxyuridine (19). Cytoplasmic RNA synthesis was detected in activated HCV positive PBLs from two HCV positive donors (Fig. 23C and 28). In contrast, no incorporation of BrU was detected in ActD treated PBLs from a HCV negative donor (Fig. 23D). In the absence of ActD, strong incorporation of BrU in newly synthesized RNA was detected in the nucleus (Figs. 23C and D). Taken together, our data clearly show that HCV RNA synthesis occurs in activated PBLs from IDUs.

Next, we wished to document HCV-directed translation in PBLs. Upon mitogen stimulation of HCV positive PBLs, NS3 and NS5B proteins were readily detected by Western blotting using several different antibodies (Figs. 24A-C). The quantity and kinetics of NS3 appearance was dependent on the particular procedure of stimulation (Figs. 24D and E) and the HCV carrier (Figs. 24F-H). This suggests that the kinetics of HCV protein production in stimulated PBLs is modulated by host factors. To show that the appearance of the proteins, which interact with the NS3 and NS5B antibodies, is dependent on HCV replication, we used siRNA against the core protein coding sequence (Figs. 24I-K). NS3 and NS5B levels decreased drastically following

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electroporation of the Core-siRNA in a dose-dependent manner when compared a to a non-specific unrelated RNA (Inverted 4E-T-siRNA; see Materials and Methods, below) (Fig. 24I). siRNA silencing resulted from a decrease of HCV RNA, as compared a to a non-specific RNA, as demonstrated by real-time PCR quantification (Figs. 24J, K).

The presence of core protein in the cytoplasm of activated HCV positive PBLs was further confirmed by Indirect Immunofluorescence (Fig. 25). Based on surveying 10 fields, we estimate that 1 to 3 % of the cells expressed high levels of HCV core protein. Taken together, the data demonstrate that translation of the HCV (+) strand RNA (Figs. 24 and 25 and transcription of the (-) strand RNA (Fig. 23) occur in activated PBLs.

To examine whether HCV particles are produced and released into the culture medium, the supernatant from PBLs was harvested and sedimented by centrifugation through a 20% sucrose cushion. The presence of HCV particles was demonstrated by Western blotting with an anti-core monoclonal antibody, MAB225P (Fig. 26A). Similar results were obtained when other anti-core antibodies (monoclonal 515S (20) and polyclonal RR8) were used (data not shown). Viral RNA co-sedimented with the HCV core protein as demonstrated by nested RT-PCR (Fig. 26B). PBLs were stimulated by methods B, P and PS (detailed in Materials and Methods) and genomic RNA isolated from the cell supernatant was quantified by real time RT-PCR (Fig. 26C). Consistent with the protein data shown above, the amount of viral RNA in the cell supernatant varied among the different stimulation procedures (Fig. 26C). To further support the evidence for

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virus production, particles were examined following metabolic labeling with ^{35}S -methionine/cysteine (Figs. 26D-G). Particles were sedimented through a 20% sucrose cushion, resuspended and floated on Optiprep™ density gradients (21) (Fig. 26D). The sedimentation range of the labeled particles (1.13-1.215 g/ml) was similar to that reported by others (22-28). HCV-E2 protein was present in the particles as determined by Western blotting using monoclonal anti-E2 1864 (Fig. 26E). The absolute quantity of HCV (+) strand RNA present in each fraction was determined by real-time RT-PCR (Fig. 26F). The HCV genomic RNA and E2 co-sedimented through the density gradient (Fig. 26F). Interestingly, Western blotting revealed that the HCV core protein sedimented throughout the gradient (data not shown). To further examine this behavior fractions 1-4 and 5-11 from the gradient were pooled and the presence of HCV E2 and core proteins was determined. The high (H) density complexes (1.111 to 1.215 g/ml) contained E2 and core protein and are likely to represent viral particles, while the low (L) density complexes (1.006 to 1.1 g/ml) contained only core (Fig. 26G). The biological significance of this observation is not immediately clear. However, it was suggested earlier that different types of particles are found in serum from chronically infected individuals (23, 29), and in the supernatant of cells expressing the full length HCV RNA (21). RNA and proteins were isolated following metabolic labeling with ^{35}S -methionine/cysteine or ^{32}P -orthophosphate (the latter in the presence of ActD) to determine whether the viral proteins and genomic RNA isolated from the different fractions was synthesized *de novo*. Supernatant was collected after labeling (Fig. 26H). Significantly, labeled RNA and

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proteins co-sedimented through the density gradient (Fig. 26H). Thus, the results show that virus particles containing *de novo* synthesized proteins and genomic RNA were released to the supernatant.

HCV particles released from HCV positive PBLs are infectious.

5 It was highly pertinent to examine whether the HCV particles released from stimulated PBLs are infectious. As it is impossible to estimate the real ratio of infectious to non-infectious virus particles produced by activated PBLs, a co-culture strategy, in which two different cell types in two chambers are separated by a 0.45 μ m
10 polyethylene terephthalate track-etched membrane, was used (Fig. 27A). The HTLV-1 transformed T cell line, MT-4 was chosen as the target cell of infection (30-33). Total RNA was extracted from infected cells and the quantity of HCV RNA was determined. Strikingly, viral
15 RNA (average of 1600 copies/ μ g of total RNA; as determined by real-time RT-PCR, data not shown) and NS3 protein were detected in MT-4, upon co-culture with activated PBLs (Fig. 27B), demonstrating that the released viral particles are infectious and that cell-to-cell contact is not required for infection. No viral proteins were detected in MT-4 cells when co-cultured with PBLs from two HCV negative donors (Fig. 27B).

20 In conclusion, we demonstrated that robust HCV replication occurs in PBLs. Without being limited to a particular theory, our success in showing replication, while earlier studies failed, can be attributed to two important factors: activation of the PBLs and the use of IDU donors. IDUs were selected because they experience a long-term altered
25 immune response (34-36) and HCV replication in PBLs has been associated with induced immunodeficiencies (37-39). Drugs have a

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variety of effects on the immune system including suppressed cell-mediated immunity (34-36). This is reflected in a depressed level of T-dependent antibody production by B lymphocytes and in an alteration of T lymphocyte function. The clinical consequences of this suppression include an increase in the incidence of viral infections such as HIV and HCV (40-42). Thus, our observations support the notion that immunosuppression in combination with cell activation act as "cofactors" in HCV pathogenesis. Studies including HCV infected individuals who are not IDUs and non-IDU immuno-suppressed individuals are required to support this hypothesis.

It is most probable that HCV enters lymphocytes during the primary infection and remains latent in resting cells. Viral latency is well documented for Epstein-Barr virus (EBV), which remains dormant in quiescent host B-cells, but enters a lytic replication phase once the cell is activated (43, 44). Interestingly, EBV can also infect T cells (45, 46). Therefore, a number of intriguing parallels can be drawn between the HCV and EBV life cycles. It is conceivable that like in EBV infection, T cell immunity plays a critical role in limiting the number of HCV infected PBLs and that during a sustained immunodeficiency state, such as that manifested in IDUs, clonal proliferation of virus infected cells will be favored. Most importantly, in this report we describe a simple cell-based system that supports robust HCV replication. The implications of these findings are paramount for several reasons. First, they clearly implicate PBLs in HCV pathogenesis. Second, they provide a model that should be useful in the quest to gain understanding of the HCV life cycle, host-virus relationship, viral infectivity and in the discovery and validation of

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novel anti-HCV agents. For the latter purpose we have established EBV-transformed B-cell lines from HCV-infected donors which should facilitate the discovery of anti-HCV drugs (see below).

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EXAMPLE 2

Materials and Methods

Antibodies. NS3 rabbit anti-serum-RB was provided by Dr. R. Bartenschlager, Department of Molecular Virology, Institute of Hygiene, University of Heidelberg, Germany. Monoclonal anti-NS5B, 5B-3B1 was
10 from Dr. D. Moradpour, Department of Medicine II, University of Freiburg, Germany. Monoclonal anti-NS3 antibody, 1G3D2 and polyclonal anti-NS3, K135 were from Dr. D. Lamarre (Boehringer Ingelheim Canada Ltd). Monoclonal anti-E2 1864 (450-470AA),
monoclonal anti-5B 10 (IFA), monoclonal anti-Core 515S (20-40AA),
15 and Core rabbit anti-serum RR8 were developed in The Tokyo Metropolitan Institute of Medical Science. Monoclonal anti-Core (Cat.No.: MAB255P; Lot:hcv-core-2-4) was purchased from Maine Biotechnology services, Inc. Monoclonal anti-human F-Actin (ab205)
was purchased from Abcam Limited. Monoclonal anti-human β -Actin
20 (clone AC-15) was purchased from Sigma-Aldrich CO. Anti-Bromodeoxyuridine monoclonal antibody-Alexa fluor 488 conjugated, and goat anti-rabbit Alexa fluor 594 conjugated were purchased from Molecular Probes, Inc..

Blood Donors and lymphocyte purification. Participants were
25 recruited through the drug addiction unit of the Saint-Luc Hospital of the Centre Hospitalier de l'Université de Montréal (CHUM) and the Saint-

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Luc Cohort study. Donors provided a written informed consent approved by the CHUM Review Board before having their blood drawn. Individuals from both sexes (87% males) were enrolled in this study between 2001 and 2003. Their mean age was 42.1 years (sd \pm 8.8) and the average time since their first injection was 16.5 years (sd \pm 9.6). 80% of the donors reported injecting drugs during the 6 month period before blood was withdrawn for this study. Cocaine and opiates were the most frequently used drugs, with 77% and 34.6% use, respectively. All HCV positive donors tested positive in a serological screen for HCV antibodies performed in the laboratory of microbiology at Saint-Luc Hospital of the CHUM using two Enzyme Linked Immunosorbent Assays (ELISA, AxSym and Cobas). Presence of HCV was confirmed by HCV-RNA detection when ELISA data were discordant. All participants recruited for this study were HIV-1 and HIV-2 negative. Serological screening for HIV antibodies was performed in the microbiology laboratory at Saint-Luc Hospital, CHUM, with an enzyme-linked immunosorbent assay (ELISA). Similar procedures were used to verify the HCV negative donors. HCV negative donors (six) were recruited from the different participating laboratories as well as from the support staff responsible for the St. Luc Cohort. Peripheral blood (20 ml) was collected from HCV positive IDU or HCV negative donors into EDTA-containing Vacutainer tubes (Becton Dickinson). Polymorphonuclear leukocytes and red blood cells were separated by centrifugation over a density gradient (Lymphocyte separation medium, cellgro®). Monocytes were then removed by plastic adherence under serum free conditions as described in The Current protocols of

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Immunology. When required, cells were frozen in 10% DMSO containing FCS and stored at -80°C prior to monocyte separation. Total PBLs were cultured in 24-well plates at 1×10^6 cells per ml in RPMI 1640 supplemented with 10% heat-inactivated FCS and antibiotics.

5

PBLs stimulation. Mitogens were added to the media (RPMI 1640, 10% FBS, and antibiotics) upon starting the culture and maintained throughout the experiment. The protocols used for PBCLs stimulation were as follows: Method A, PBLs were grown in the presence of irradiated L4.5 cells (murine fibroblasts expressing the CD40 ligand, CD154) as described (49). Method B, $1 \mu\text{g/ml}$ of anti-CD3 and 200 U/ml of IL-2 (Sigma-Aldrich CO) were added. Method P, $3 \mu\text{g/ml}$ phytohemagglutinin (PHA, Sigma-Aldrich CO), and 200 U/ml IL-2 were used. Method PS, $1:10^4$ vol/vol of Staphylococcus aureus Cowan fixed cells (SAC, Calbiochem) in combination with phytohemagglutinin and 200 U/ml IL-2 were added to the media. Method S, $1:10^4$ vol/vol of SAC and 200 U/ml of IL-4 (Sigma-Aldrich CO) were used. Cell activation was verified by flow cytometry. Cells were rinsed twice with 1 ml cold phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , pH 7.4) and fixed in 80% ethanol/PBS for 30 min at 4°C . PBS (2 volumes) was added and cells were pelleted by centrifugation. Cells were rinsed twice with 2 ml PBS and then resuspended in 0.5 mL PBS containing $0.2 \mu\text{g/ml}$ RNase A and incubated for 40 min at 37°C . Propidium iodide was added to a final concentration of $1.2 \mu\text{g/ml}$ and samples were analyzed by flow cytometry using a single laser FACS instrument (Becton-Dickinson)

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combined with the CellQuest™ software.

RNA purification. Total RNA was extracted from cells using Trizol™ (Invitrogen) according to the manufacturer's protocol. Yeast tRNA (1 mg/ml) was added as a carrier. RNA was resuspended in nuclease-free water (Sigma-Aldrich CO). Total RNA was quantified by PhosphorImager™ (STORM system, Molecular Dynamics) using the RiboGreen™ RNA Quantification Kit (Molecular Probes, Inc).

- 10 **Nested RT-PCR.** HCV-RNA was detected in cells by a reverse transcription-polymerase-chain reaction (one step RT-PCR reaction, 45 cycles, Qiagen) against the highly conserved 5' untranslated region (sense primer from nucleotide 13 to 38 and the anti-sense primer from nucleotide 383 to 359) of the HCV genome (strain H77 pCV-H77C, EMBL:AF011751, MEDLINE: 97385173) followed by a second round of amplification, nested PCR (45 cycles, sense primer from nucleotide 59 to 82 and the anti-sense primer from nucleotide 307 to 285, strain H77 pCV-H77C) using Taq DNA polymerase (MBI Fermentas). β -Actin was amplified (30 cycles) using the sense primer 5'-GTGGGGCGCCCCAGGCACCA-3' and antisense primer 5'-GTCCTTAATGTCACGCACGATTTC-3'.

- 25 **Real Time RT-PCR.** Two methods were used to detect and quantify HCV RNA. Method I: Reverse transcription was carried out at 50°C for 20 minutes in a one-tube two-step RT-PCR reaction with Thermoscript™ reverse transcriptase (Invitrogen), 10 μ M of HCV-

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tagged strand-specific RT primer and 100 μ M of anti-sense GAPDH primer (Table S2). The reverse transcriptase was inactivated by heating for 5 minutes at 95°C and PCR (22 cycles) with Platinum Taq DNA polymerase was performed in a Trio-thermocycler™ (Biometra): at 94°C for 45 s, 60°C for 60 s, 68°C for 2 min. The first round PCR products were then amplified for 40 cycles in the Roche LightCycler™ instrument: denaturation at 95°C for 60 s, and amplification and quantification at 95°C for 15 s, 60°C for 10 s with a single fluorescence measurement, 72°C for 15 s. Real-time quantification of RNA copy numbers for HCV and the human GAPDH gene was based on a set of eight log₁₀ external standards covering 10⁸ to 10¹ plasmid copies of a pCRII vector containing the 5' HCV leader (*genotype 1a*) and the GAPDH normalization PCR amplicons which were run in parallel with the test samples. RNA extracted from PBLs of a HCV negative donor was used as control. As a reaction control for the strand-specific signal, the RT step of the RT-PCR was carried out without a HCV-tagged primer. The presence of HCV non-structural proteins in the cell samples used for RNA preparation was confirmed by Western blotting (data not shown). Method II: Real-time RT-PCR was performed on the ABI Prism 7700 Sequence Detection System using the TaqMan EZ RT-PCR Kit (Applied Biosystems). RNA sample (5 μ l), combined with 45 μ l of Reagent Mix, was used for the Real-Time RT-PCR reaction. In vitro transcribed replicon RNA was used as a standard to determine HCV copy numbers (1 μ g of replicon RNA equals 2.15x10¹¹ HCV copies). The RNA copy number was normalized (RiboGreen RNA quantification, Molecular Probes Inc.) and expressed as genome equivalents per ml of

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total supernatant.

Bromo-uridine labeling: Bromo-uridine (BrU, 5-Bromouridine 5'-Triphosphate, Sigma-Aldrich CO) was incorporated into PBLs using a modified version of the procedure of Haukenes et al. (50). BrU (10 mM)
5 was incubated with an equal volume of Lipofectamine™ 2000 transfection reagent (Invitrogen) for 30 min at room temperature and added to 250 µl of cells resuspended in optimem medium (Invitrogen) in a 1:1 (vol/vol) ratio. The BrU/Lipofectamine™ 2000 mixture was added to cells 6 h after activation. Cells were incubated for 5h, washed and
10 resuspended in mitogen (method P) containing culture medium. Cells were collected after a 12 h incubation period at 37 °C in a 5% CO₂ environment. When actinomycin D (ActD, Sigma-Aldrich CO) was used, cells were incubated with the drug (5 µg/ml) starting 30 min prior to the addition of BrU. ActD was maintained throughout the experiment.

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Immunofluorescence. Immunofluorescence was performed on 5x10⁴ cells. Following cytopsin for 7 min at 1100 rpm in a Cytosin 2 (Shandon), cells were dried for 30 min at room temperature and fixed for 30 min at -20°C in a mixture of acetone and methanol (1:1 vol/vol).
20 Cells were blocked for 30 min at room temperature in 10 mM Tris-HCl pH 8.0 containing 1% BSA. Slides were washed 3 times with PBS and incubated at room temperature for 2 h with the polyclonal anti-core RR8 antibody (1/50) or overnight at 4°C with the anti-bromo-deoxyuridine Alexa Fluor 488 conjugate antibody (2 µg/ml) in a humidified box.
25 were washed 3 times with PBS. For Core detection, slides were

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Incubated 1 h at room temperature with an Alexa-594 conjugated antibody (dilution 1/250). DAPI staining was performed for 7 min at room temperature (1 μ g/ml final concentration). Mounted slides (Permout mounting medium, Fisher Scientific) were stored overnight at 4°C prior to analysis. Conventional epifluorescence micrographs were obtained on a Zeiss Cell Observer system equipped with an Axiovert 200 M microscope using the 100X oil lens. Images were digitally deconvoluted with the AxioVision 3.1 software using the Nearest Neighbor deconvolution method that uses the Agard's formula.

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Western Blots. Proteins extracts were prepared by sonification in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl pH 7.5) and quantified (BSA assay, BioRad). Proteins (10 μ g of extracts from PBLs or 5 μ g of extract from Huh7 cells stably expressing the HCV replicon (47)) were resolved on SDS-10% polyacrylamide gels (PAGE) and transferred to 0.2 μ m Protran nitrocellulose membrane (Schleider and Schuell) for 1 h at 100V. The membrane was blocked with PBS containing 0.5% Tween-20 (PBS-T) and 5% nonfat dry milk. Blots were then incubated with the primary antibody for 2 h at room temperature, washed 3 times with PBS-T and incubated for 1 h with a horse radish peroxidase (HRP) conjugated secondary antibody. Blots were visualized using an enhanced luminol reagent (ECL; PerkinElmer Life Sciences Inc).

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Radio labeling and gradient purification of virus particles. A total of 1×10^6 activated PBLs were first preincubated in methionine- or

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phosphate-free RPMI for 30 min, and then incubated for 12 h in the same media supplemented with [³⁵S] protein labeling mix (1175 Ci/mmol) or carrier-free inorganic ³²P (500 µCi/ml, H₃PO₄, ICN Biomedicals, INC), the latter in presence of ActD (5 µg/ml). Supernatant
5 was collected, cells and cellular debris was removed by low-speed centrifugation at 1600 x g for 15 min at 4°C, followed by filtration with 0.45 µm pore size filter (Fisherbrand, Fisher scientific). Particles were partially purified by ultracentrifugation through a 20% sucrose cushion for a minimum of 6 h at 4°C (in Beckman L8-55 ultracentrifuge) at
10 35,000 rpm in a SW-41 rotor. Sediments were resuspended in serum free RPMI and Iodixanol (Optiprep™, Invitrogen) was added to a final concentration of 40% w/v (ρ=1.216). The sample was laid over a 60% wt/vol Optiprep™ solution (ρ=1.320 g/ml) and then overlaid with a linear iodixanol gradient (ρ=1.038 to 1.205 g/ml) prepared in RPMI and spun
15 for 20 h at 4°C in Beckman L8-55 ultracentrifuge at 30,000 rpm using a SW-41 rotor. Fractions were collected from the top of the tube and RNA was prepared as described above. Half of the final RNA volume was mixed with liquid scintillation cocktail (EcoLite™, ICN Biomedicals) and ³²P radioactivity was counted in a Beckman LS 6500 scintillation
20 counter. Proteins were extracted by directly adding 10X RIPA buffer to a final concentration of 1X RIPA. 1/100th of the protein extract was mixed with liquid scintillation cocktail and ³⁵S radioactivity was determined using a Beckman LS 6500 scintillation counter. 1/10 of the protein extract was directly mixed with concentrated Laemmli sample
25 buffer, resolved on a SDS 15%-PAGE, and transferred to 0.2 µm

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Protran nitrocellulose membrane over night at 30V. The membrane was dried and exposed against Kodak Biomax™ MR film. The remaining protein extract was concentrated by TCA precipitation (15% final). Proteins were washed twice with ether, dried and dissolved in a solution containing 3 M urea, 26 mM EDTA (pH 8), and 0.5 µg/ml of RNase A. Samples were mixed with concentrated Laemmli sample buffer, resolved on a SDS 10% PAGE and transferred to 0.2 µm Protran nitrocellulose membrane for 1 h at 100V. Proteins were detected by Western blotting as described above.

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siRNA. The target sequence for the siRNA was chosen using the Ambion web-based criteria. The selected RNA oligonucleotides, Core (from nucleotide 371 to nucleotide 391, strain H77 pCV-H77C, EMBL:AF011751, MEDLINE: 97385173) and the unrelated non-specific RNA (inverted sequence for 4E-T from nucleotide 986 to nucleotide 1008; DDBJ/EMBL/GenBank database, accession No. AF240775), were synthesized by Dharmacon Research (Lafayette, CO) and handled according to the manufacturer's instructions. Varying amounts (3 µl or 5 µl of a 20 µM solution) of RNA duplexes were electroporated using a Gene pulser® II electroporator (BioRad), into 1×10^6 PBLs in 0.5 ml of serum free RPMI. Cells were treated with a pulse of 975 µF and 300 V. Then 0.5 ml of RPMI containing 20% FCS was added and the cells were seeded in a 24-well cell culture dish. Protein and RNA extracts were harvested 48 h after electroporation. Immunoblots were performed as described above using an NS3 rabbit antiserum-RB and monoclonal anti-NS5B, 5B-3B1. HCV RNA levels were quantified by

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real-time RT-PCR using method I.

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Table S1. Characteristics of the IDU donors, enrolled between March 2001 and April 2003:

Participant	Age (years)	Sex	IDU duration (years)	Under Methadon e treatment	IDU (past 6 months)	Opioids excl methadone (past 6 months)	Cocaine (past 6 months)
SB-1	41	male	22	yes	no	no	no
SB-2	42	female	20	yes	yes	yes	yes
SB-4	35	male	11	yes	yes	yes	yes
SB-5	21	female	3	yes	yes	yes	no
SB-6	32	male	1	yes	yes	yes	yes
SB-7	45	male	18	yes	no	no	no
MLL 001	48	male	31	no	yes	yes	yes
MLL 002	39	male	3	no	yes	no	yes
MLL 003	38	male	10	no	yes	yes	yes
MLL 004	47	male	32	no	yes	yes	yes
MLL 005	38	male	21	no	yes	no	no
MLL 006	49	male	37	yes	yes	yes	no
MLL 007	61	male	36	no	yes	no	no
MLL 008	39	male	13	no	no	no	yes
MLL 009	23	male	5	no	yes	no	no
MLL 010	40	male	21	no	no	no	yes
MLL 011	45	male	6	no	yes	no	yes
MLL 012	48	male	14	no	yes	yes	yes
MLL 013	49	male	24	no	no	yes	no
MLL 014	41	male	18	no	yes	no	yes
MLL 015	38	male	6	no	yes	yes	yes
MLL 016	34	male	11	no	no	no	no
MLL 018	42	male	13	no	yes	no	yes
MLL 019	51	male	10	no	yes	no	yes
MLL 020	38	male	13	no	yes	no	yes
MLL 021	35	female	5	no	no	no	no
MLL 022	43	male	29	no	yes	no	yes
MLL 023	52	male	20	no	yes	no	yes
MLL 024	37	male	13	no	yes	no	yes
MLL 025	36	male	18	yes	yes	yes	yes

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Participant	Age (years)	Sex	IDU duration (years)	Under Methadon e treatment	IDU (past 6 months)	Opioids excl methadone (past 6 months)	Cocaine (past 6 months)
MLL 026	29	female	13	yes	yes	yes	yes
MLL 027	52	male	11	no	yes	yes	yes
MLL 028	45	male	6	no	yes	yes	yes
MLL 029	42	male	6	no	yes	yes	yes
MLL 030	43	male	10	no	yes	no	yes
MLL 031	36	male	19	yes	yes	no	yes
MLL 032	22	male	11	yes	yes	yes	no
MLL 033	24	male	7	yes	yes	yes	yes
MLL 034	52	male	26	no	yes	no	yes
MLL 035	61	male	36	no	yes	no	no
MLL 036	49	male	31	no	yes	no	yes
MLL 037	57	male	36	no	no	no	no
MLL 038	27	male	11	no	yes	yes	yes
MLL 039	42	female	17	yes	yes	yes	no
MLL 040	53	male	40	no	yes	yes	yes
MLL 041	34	male	11	no	no	no	yes
MLL 042	47	male	7	no	yes	no	yes
MLL 043	42	female	23	no	no	no	no
MLL 044	30	male	11	no	no	no	yes
MLL 045	41	male	22	no	yes	no	yes
MLL 046	43	male	21	no	yes	yes	yes
MLL 047	41	male	18	no	yes	no	yes
MLL 048	47	male	22	no	yes	no	yes
MLL 049	52	male	11	no	no	no	yes
MLL 050	33	male	10	no	yes	no	yes
MLL 051	45	male	30	yes	yes	no	yes
MLL 052	33	male	8	no	yes	no	yes
MLL 053	43	female	12	no	yes	no	yes
MLL 054	46	male	22	no	yes	no	yes
MLL 055	36	female	21	yes	yes	no	yes
MLL 056	40	male	14	no	no	no	yes
MLL 057	37	male	9	no	yes	yes	yes
MLL 058	45	male	30	yes	yes	no	yes
MLL 059	50	male	30	no	yes	yes	yes

-43-

Participant	Age (years)	Sex	IDU duration (years)	Under Methadon e treatment	IDU (past 6 months)	Opioids excl methadone (past 6 months)	Cocaine (past 6 months)
MLL 060	35	male	12	yes	yes	yes	no
MLL 061	46	male	7	no	no	no	yes
MLL 062	48	male	11	yes	yes	yes	yes
MLL 063	66	female	35	no	yes	no	yes
MLL 064	38	male	3	no	yes	no	yes
MLL 065	33	male	10	no	yes	no	yes
MLL 066	48	male	11	yes	no	no	no
MLL 067	46	male	11	no	yes	no	yes
MLL 068	42	male	6	no	yes	no	yes
MLL 069	42	male	23	no	yes	yes	yes
MLL 070	44	male	11	no	yes	no	yes
MLL 071	47	female	22	no	yes	no	yes
MLL 072	61	male	16	no	yes	no	yes
MLL 073	37	male	9	yes	no	no	no

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Table S2. Probes and primers used in Real Time RT-PCR method 1.

<u>Name</u>	<u>Orientation</u>	<u>Used in:</u>	<u>Target</u>	<u>Nucleotide position in target</u>
H250	sense 5' external	RT and 1st round PCR	HCV	64-84
HC110	antisense 3' external		HCV	456-475
G.24	sense 5' external		GAPDH	15-32
G.589	antisense 3' external		GAPDH	581-597
H190	sense 5' internal	real-time PCR	HCV	142-161
C40	antisense 3' internal		HCV	385-405
G.174	sense 5' internal	Hybridization Probes	GAPDH	166-182
G.511	antisense 3' internal		GAPDH	502-520
297.P1	sense FL1 probe		HCV	274-297
300.P2	sense FL2 probe		HCV	300-324
G.P1	antisense FL1 probe		GAPDH	187-212
G.P2	antisense FL2 probe		GAPDH	214-238

EXAMPLE 3

Established EBV-Transformed Cell Line enabling Robust Hepatitis
5 C virus replication in peripheral blood lymphocytes from infected
donors

It is shown herein that HCV can naturally infect blood cell and can replicate therein (Figs. 29-41). In order to assess whether the

-45-

produced HCV was infectious the protocol of Fig. 42 was followed. We show that HCV replicating in naturally infected PBLs was indeed infectious. We further went on to generate an HCV expressing cell line. In an embodiment, we developed an EBV-cell line that is able to replicate
5 HCV.

B-cells from infected donors were identified as the cells that harbored HCV virus. These cells were immortalized by EBV infection. Interestingly, when grown under normal conditions, the EBV-immortalized B cells from infected donors, do not produce detectable
10 amounts of HCV proteins. However, when stimulated (independent from the stimulation procedure P, S or PS) virus proteins (NS3 and NS5) become detectable (Fig 43).

Peripheral blood lymphocytes (PBLs) obtained from an HCV negative donor can be infected by co-culturing with stimulated
15 EBV-transformed B-cells from an HCV positive donor (Fig 44). This implies: a) PBLs are infectable, thus HCV has tropism for these cells, b) HCV produced by the EBV-transformed B-cells from an HCV positive donor is infectious.

Advantages of this system include:

20 a) EBV-transformed B-cells grow in culture. Therefore, a cell based replication system for HCV has been developed. b) EBV-transformed B-cells proliferate under normal culture conditions (RPMI 1640, Antibiotics and 10% serum), but produce the virus only when stimulated. c) the released virus is infectious. Therefore, this system can be used for HCV
25 receptor identification. d) This system should prove useful in the discovery and validation of new anti-HCV agents at all levels of the virus

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life cycle (entry, protein synthesis, RNA replication, assembly and release).

CONCLUSIONS

5 The present invention relates among other things to the fact that: (1) HCV has PBMC tropism; (2) HCV can naturally infect blood cells; (3) HCV can replicate in PBMCs and PBMLs; (4) HCV replicating in naturally infected PBMCs is infectious; (5) HCV can replicate in extrahepatic tissue; and (6) HCV has a latent phase during
10 PBMC infection, which can be ended by activation.

 It is interesting to note that HCV replication is activated upon immune response. Thus, a person of ordinary skill in the art will be able to provide other methods of activation than those disclosed herein (or complementary thereto) to activate HCV replication in PBMCs or
15 PBLCs, without undue experimentation.

 The present invention provides the tools to study hepatitis C virus replication in a simple cell culture based system. This simple culturing tool is suitable for the search and validation of novel HCV antiviral drugs and therapies (vaccine). The assays and methods
20 of the present invention enable the performance of screening assays to identify antiviral agents. Of course, the assays can be highthroughput. Compound libraries can now be used to identify candidate anti-HCV agents. These assays can thus be used to generate lead compounds for pharmaceutical anti-HCV formulations.

25 The novel replication system of the present invention, in one embodiment, based on PBMCs (or PBMLs) is simple, does not

-47-

require facilities other than those normally used for HIV research, and allows experiments with the complete HCV. Thus, novel drugs and therapies can be screened to target all the different stages of virus replication such as virus entry, cytoplasmic replication (viral (-) and (+) strand synthesis), viral protein synthesis, virus assembly, virus trafficking, and virus release.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified without departing from the spirit and nature of the subject invention as defined in the appended claims.

The present invention thus shows that: 1) HCV can replicate in extrahepatic tissue; 2) HCV has PBL tropism; 3) Virus produced by PBLs is infectious; 4) HCV has a latent phase during PBL infection; 5) EBV-transformed HCV producing B cells were generated (and thus demonstrate one means of generating established cell lines which can produce HCV); and 6) HCV latency is most probably due to the presence of an inhibitor of viral translation initiation.

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WHAT IS CLAIMED IS:

1. A simple *in vitro* culture system, which is suitable for the replication of hepatitis C virus (HCV).
5
2. The system of claim 1, which enables the replication of complete HCV.
3. A tissue culture system for HCV which enables the
10 screening and development of drugs and therapies for essentially all the different stages of virus replication such as virus entry, cytoplasmic replication [viral (-) and (+) strand synthesis], viral protein synthesis, virus assembly, virus trafficking, and virus release.
4. A method of generating a vaccine to HCV
15 comprising a pulsing of monocyte-derived dendritic cells (DCs) with HCV, co-cultured with autologous peripheral blood lymphocytes from a HCV-seropositive individual.
5. A method of activating the replication of HCV in
20 PBMCs comprising obtention of same from a HCV-infected patient and activating the replication of HCV by incubating the PBMCs with an activation-inducing amount of at least one mitogen (e.g. activator).
6. A co-culturing system for replicating HCV *in vitro*
25 which comprises co-culturing PBMCs (or PBLs) infected with HCV,

wherein the PBMCs have been activated and in which the HCV can replicate, together with a cell line, wherein the co-culturing enables infection of the cell line and replication of the HCV thereinto. In a particular embodiment of the present invention, the cell line is an
5 immortalized cell line.

7. The system of claim 6, wherein said cell line is an immortalized cell line.

10 8. An assay for screening a test agent and selecting an agent which possesses anti-HCV activity, comprising:
a) growing a HCV infected cell according to an *in vitro* assay of the present invention; and
b) assaying replication, translation, assembly infection or
15 the like of HCV.

9. A method for identifying, from a library of compounds, a compound with anti-HCV activity, comprising:
a) providing a screening assay comprising a
20 measurable biological activity of HCV;
b) contacting said screening assay with a test compound; and
c) detecting if said test compound inhibits the biological activity of HCV;
25 wherein a test compound which inhibits said biological activity is a compound with said inhibitory effect.

10. The method of claim 9, wherein the test compound
with said therapeutic effect is further modified by combinatorial or
medicinal chemistry to provide further analogs of said test compound
5 also having said therapeutic effect.

11. A compound having therapeutic effect on HCV,
comprising:
a) providing a screening assay comprising a
10 measurable biological activity of HCV;
b) contacting said screening assay with a test
compound; and
c) detecting if said test compound inhibits the
biological activity of HCV;
15 wherein a test compound which inhibits said biological activity is a
compound with said inhibitory effect.

12. The compound of claim 11, wherein the compound
with said therapeutic effect is further modified by combinatorial or
20 medicinal chemistry to provide analogs of said compound also having
said therapeutic effect.

13. The system of claim 6, wherein said cell line is an
EBV-transformed cell line.
25

14. A cell capable of enabling robust HCV expression

in co-cultured cells.

15. The cell of claim 14, wherein said cell is established and said co-cultured cells are PBLs.

5

16. A kit for producing HCV in culture comprising
a) HCV infected cells capable of producing HCV; b) non infected cells
for which HCV-produced by said cells of a) has tropism, and in which
HCV robust replication occurs; and c) a stimulating (e.g. inducing) factor
10 capable of inducing robust HCV replication in said HCV-infected cell
line.

Hepatitis C virus (HCV) genome organization.

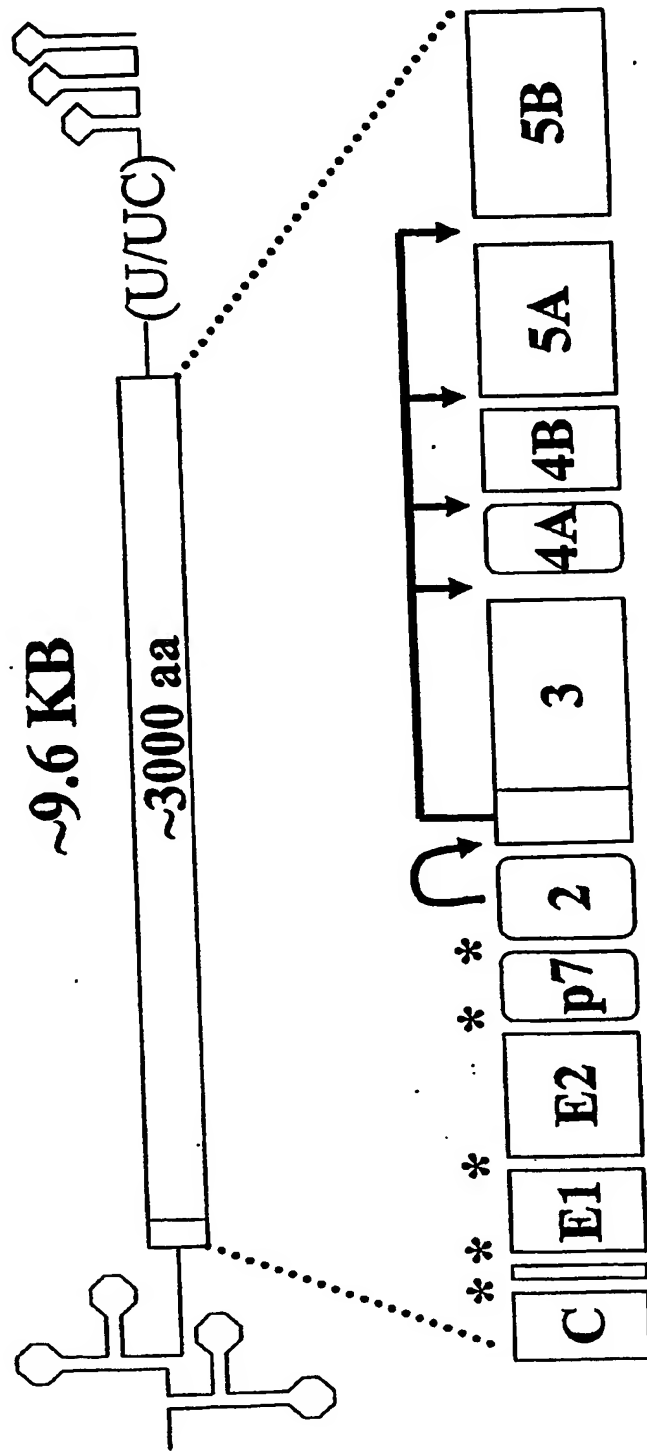


FIGURE 1

J. Gen Virol. 2000, 81, 1631.

Hypothetical model of the HCV replication cycle.

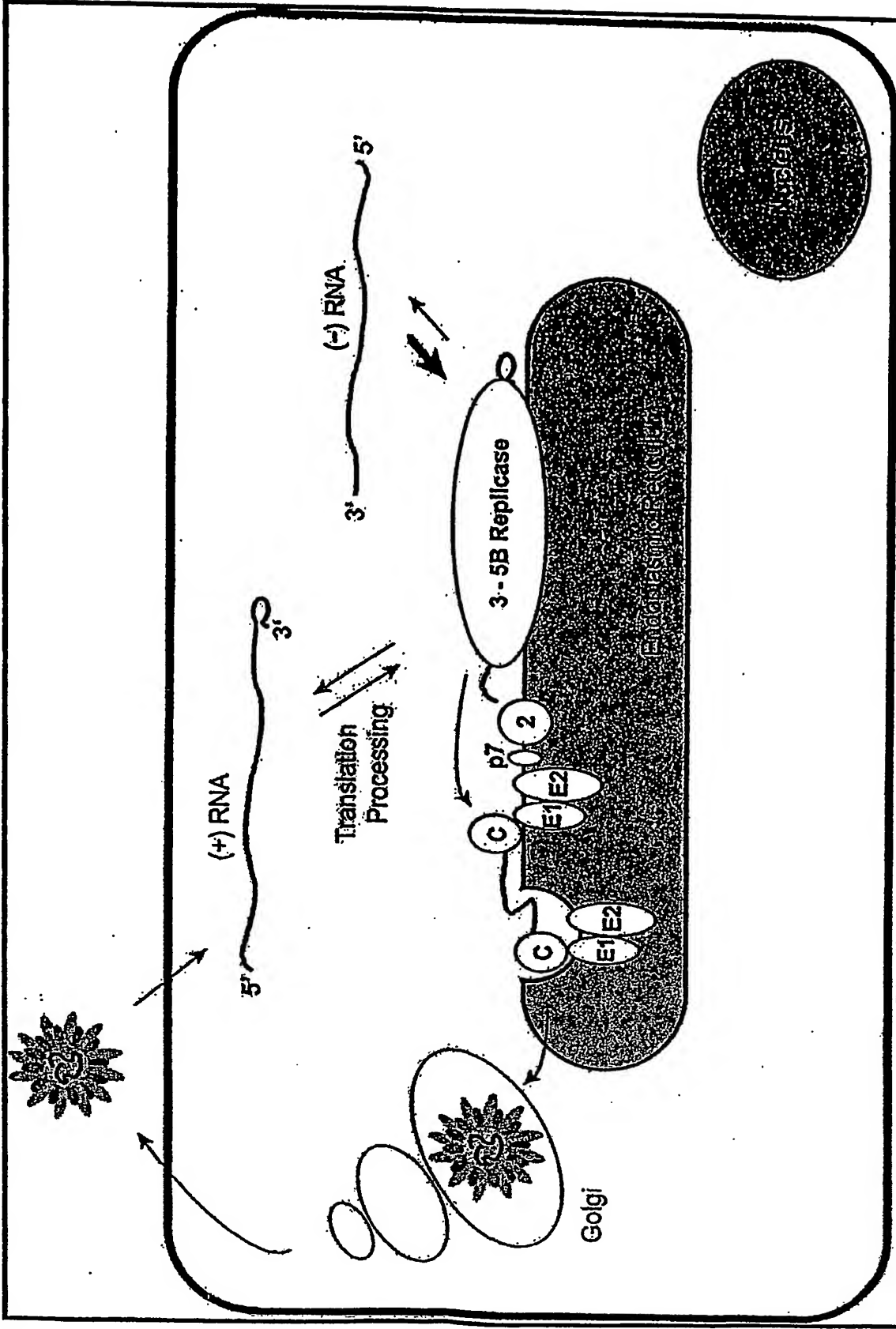


FIGURE 2

Experimental Protocol.

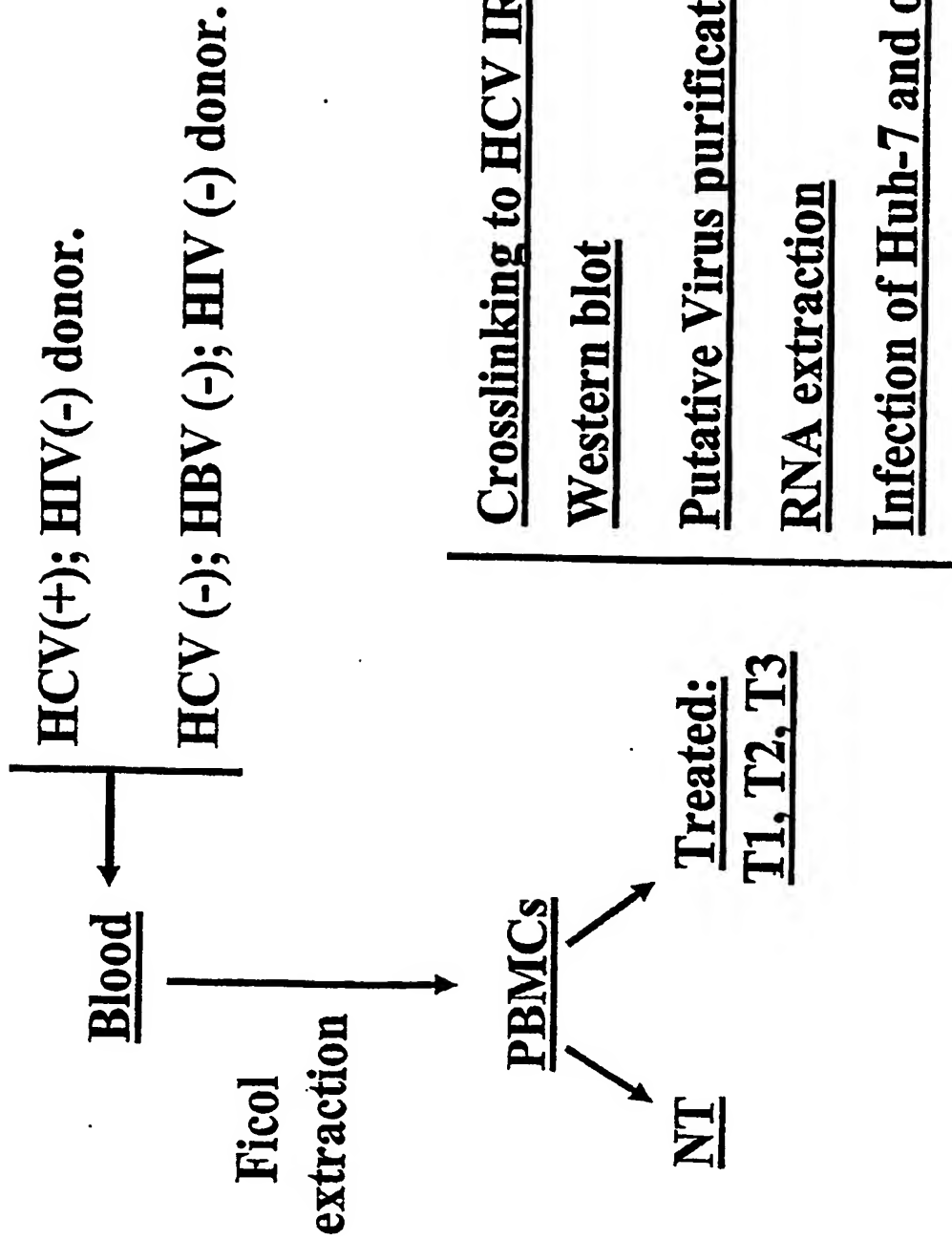
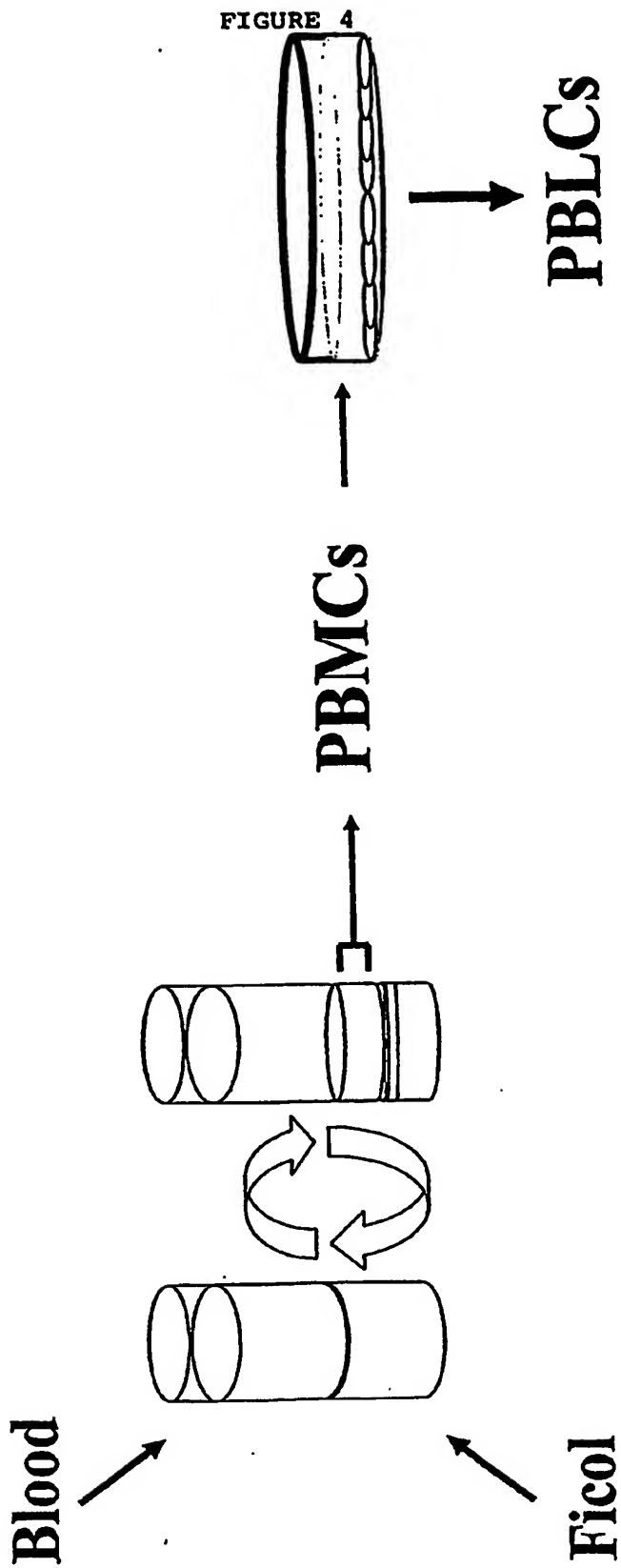


FIGURE 3

PBMC and PBLC purification from blood samples.

CA 02436104 2003-07-14



**Detection of HCV NS3 and NS5 proteins in cell extracts from
Treated PBMC from an HCV (+) patient.
[Coating anti-NS3 polyclonal antibody; Dr. Richardson anti-NS5 monoclonal antibody]**

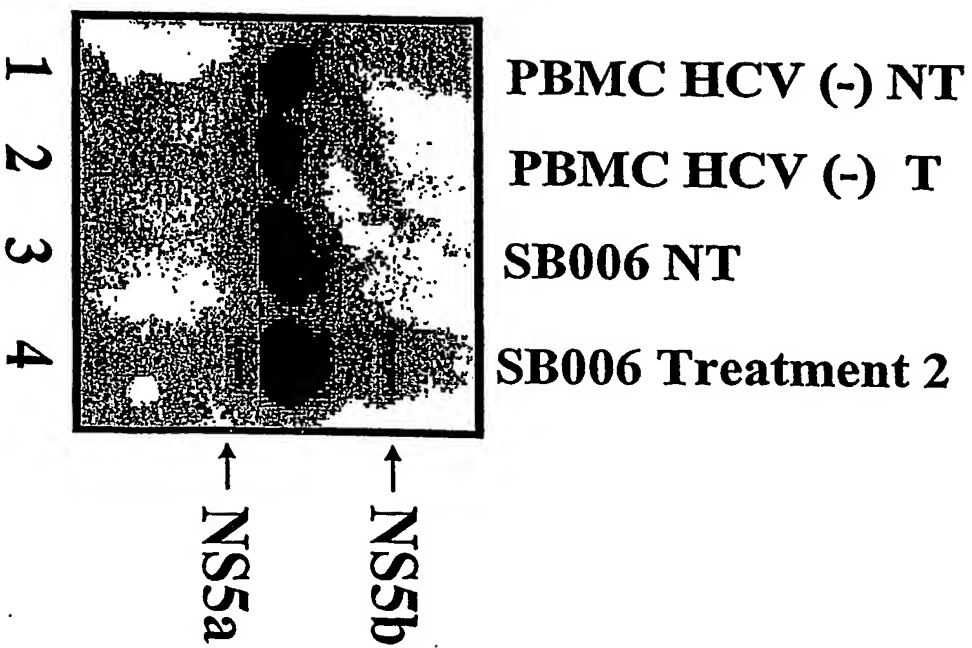
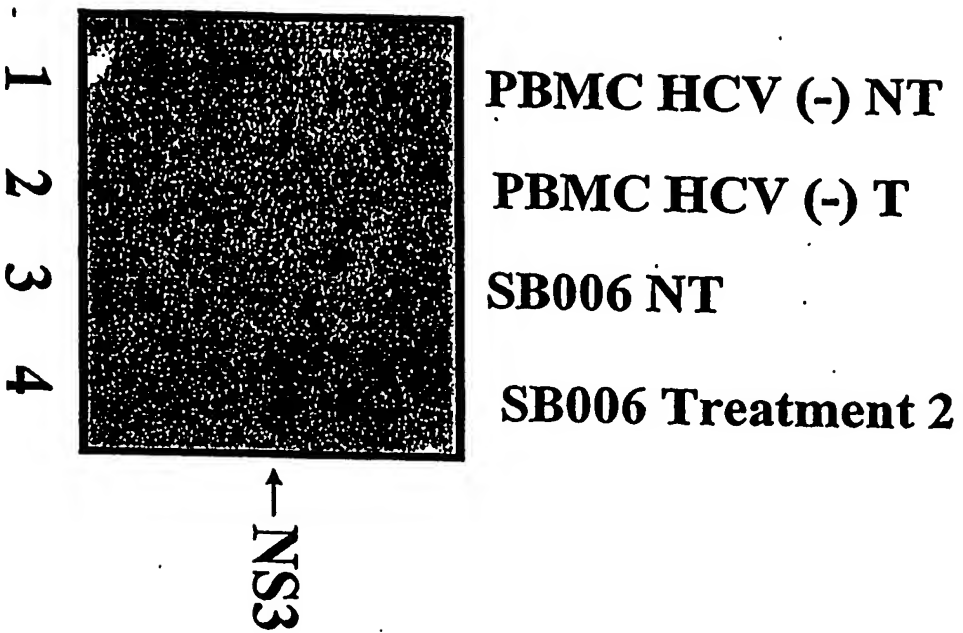
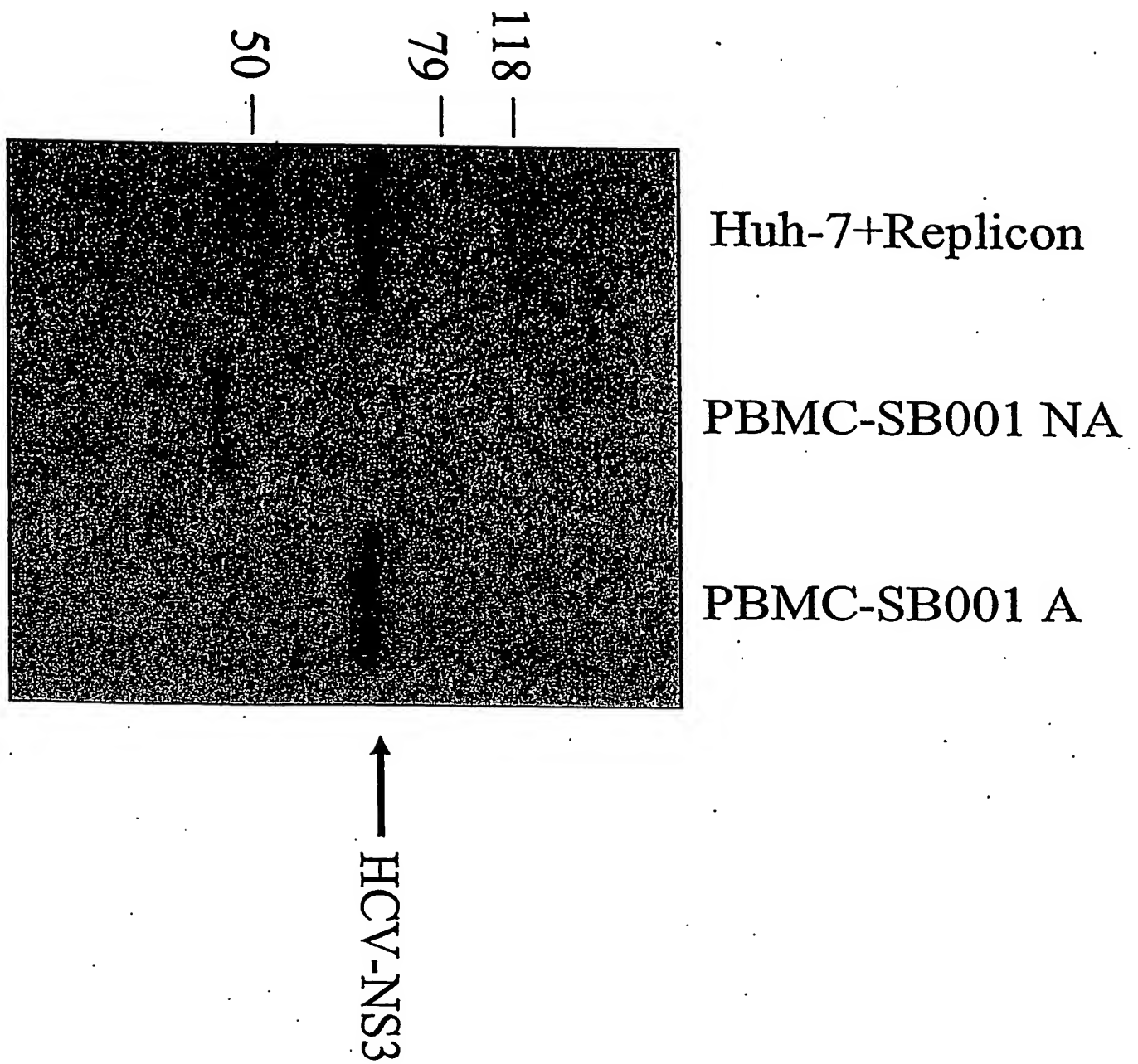


FIGURE 6



Time course of HCV-NS3 detection: PBMCs From patient MLL-001

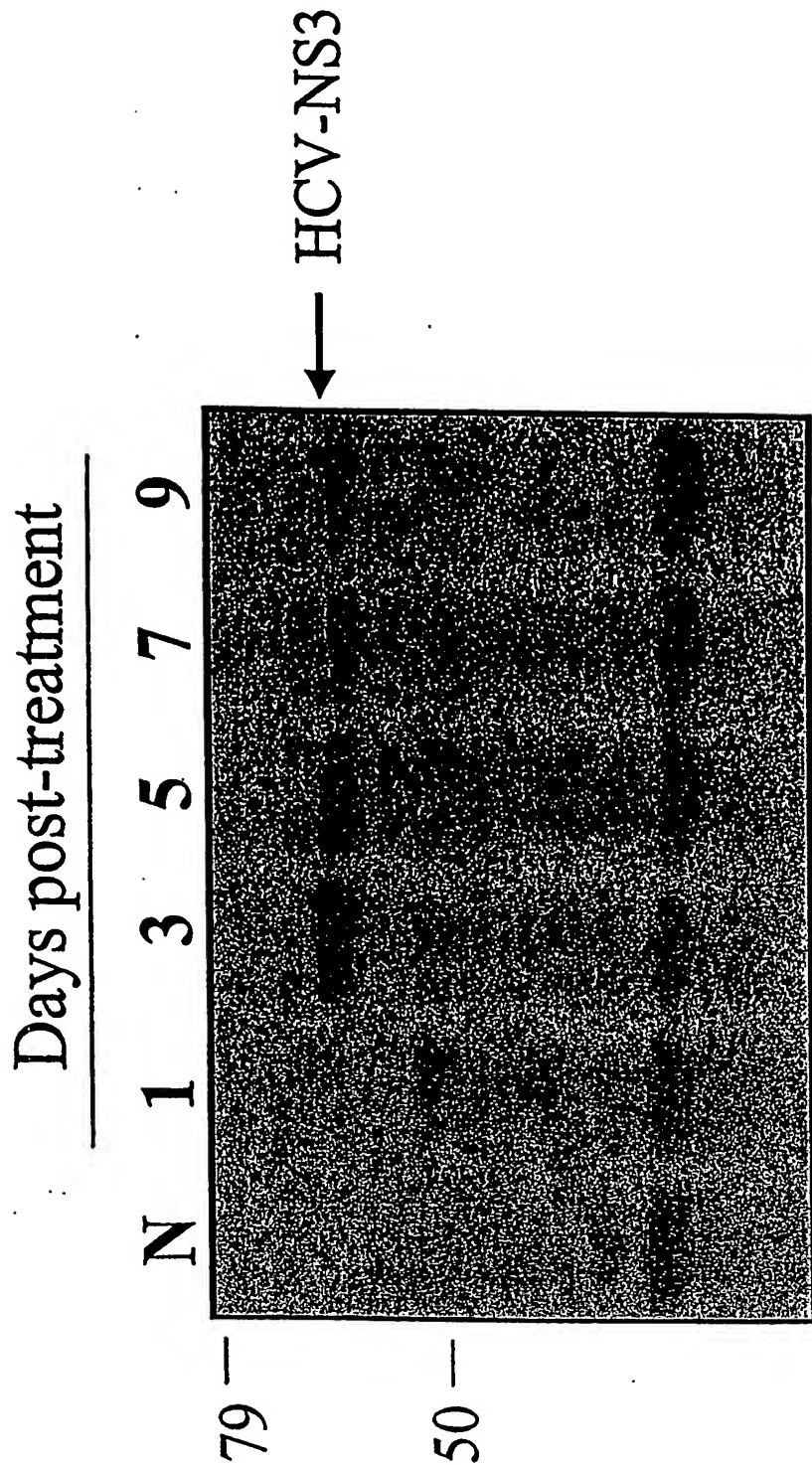


FIGURE 7

Time course HCV-NS3 detection: PBMCs from patient MLL-002

Days post-treatment

N 1 3 5 7 9

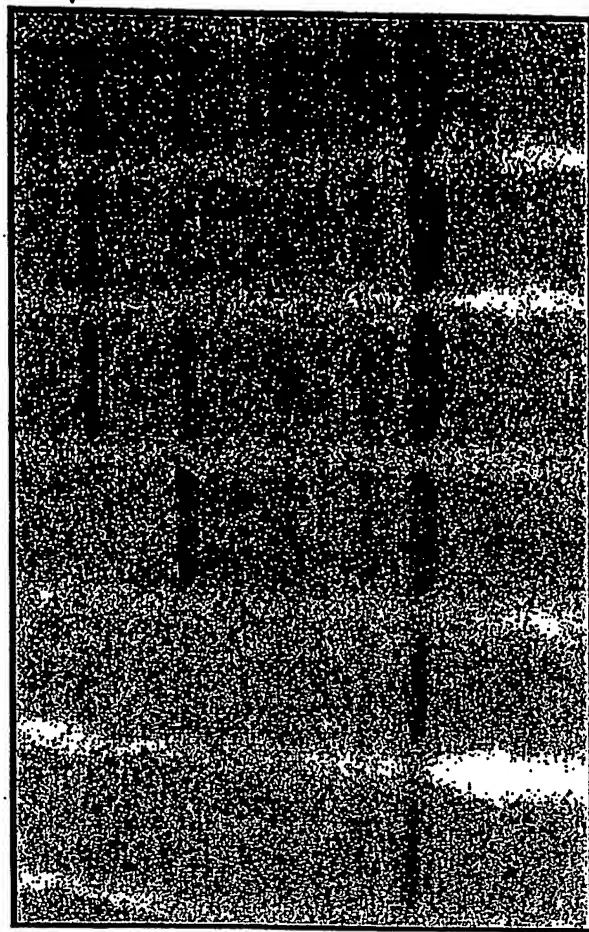


FIGURE 8

Detection of HCV-NS3 protein in treated (N3) PBMCs from HCV9+) donors

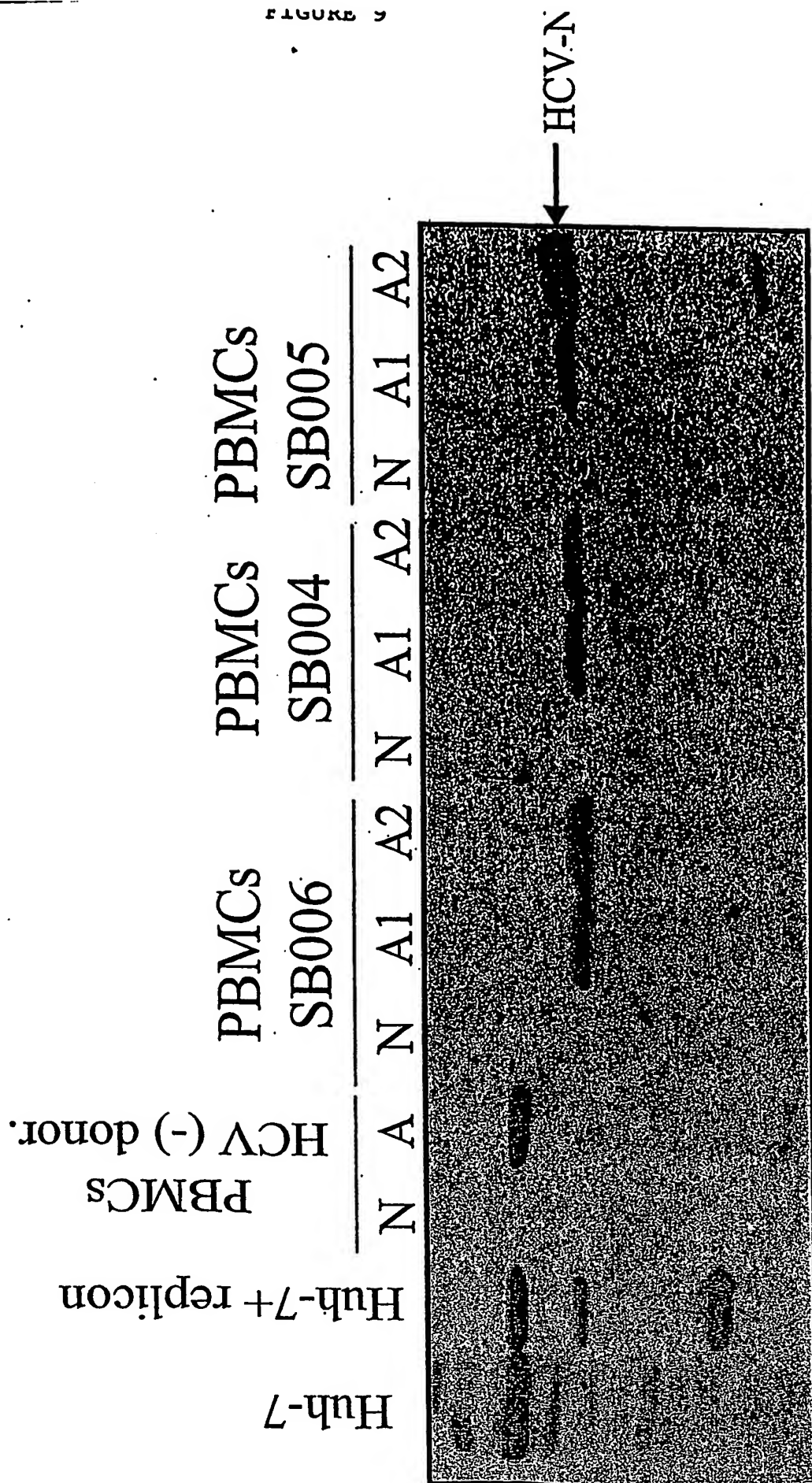
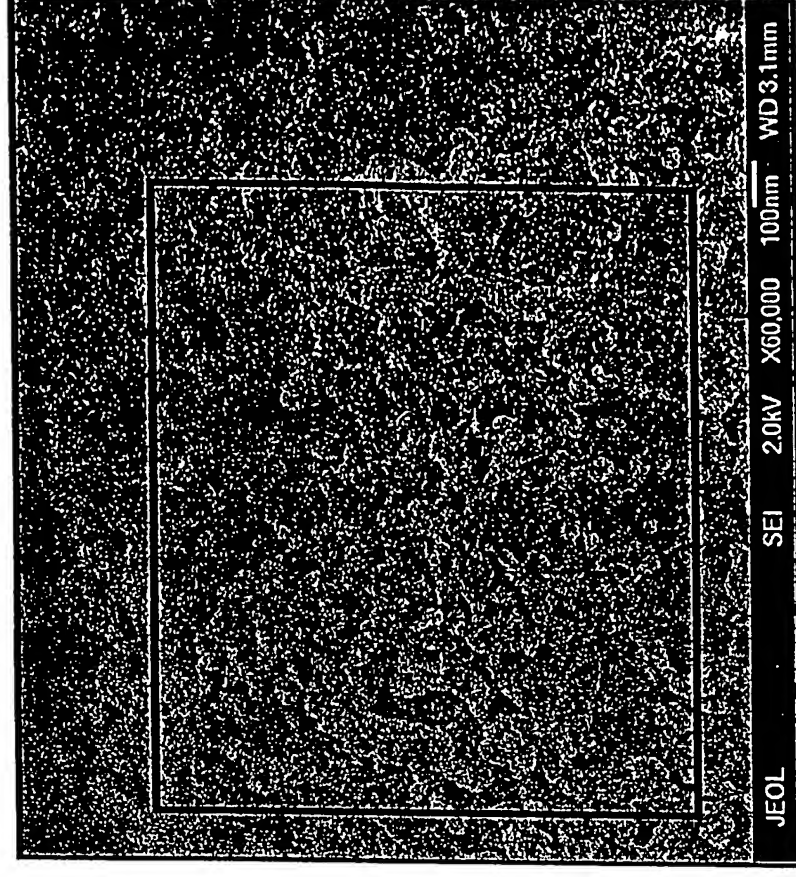


FIGURE 3

Detection of virus like particles by scanning electron microscopy

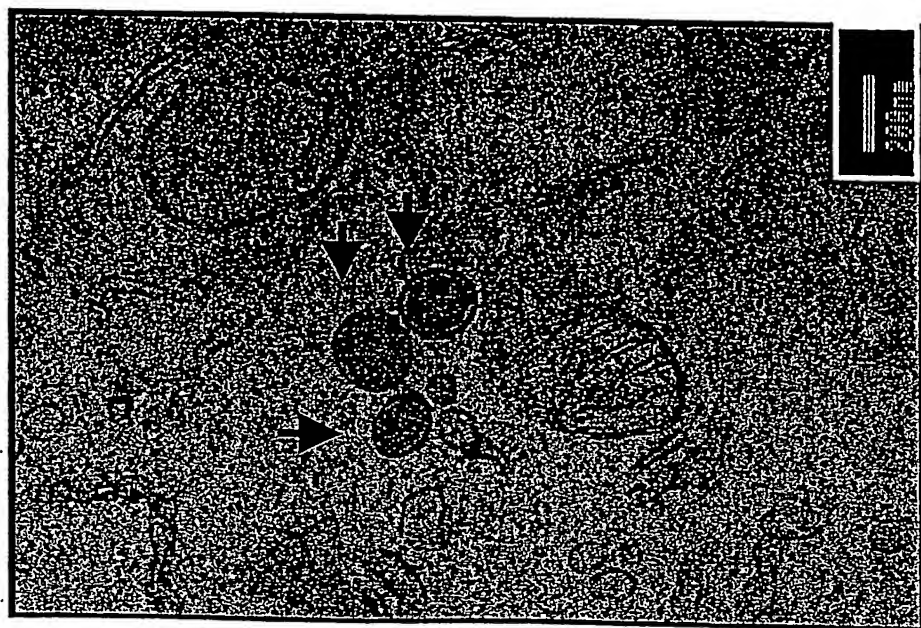


(-) Control

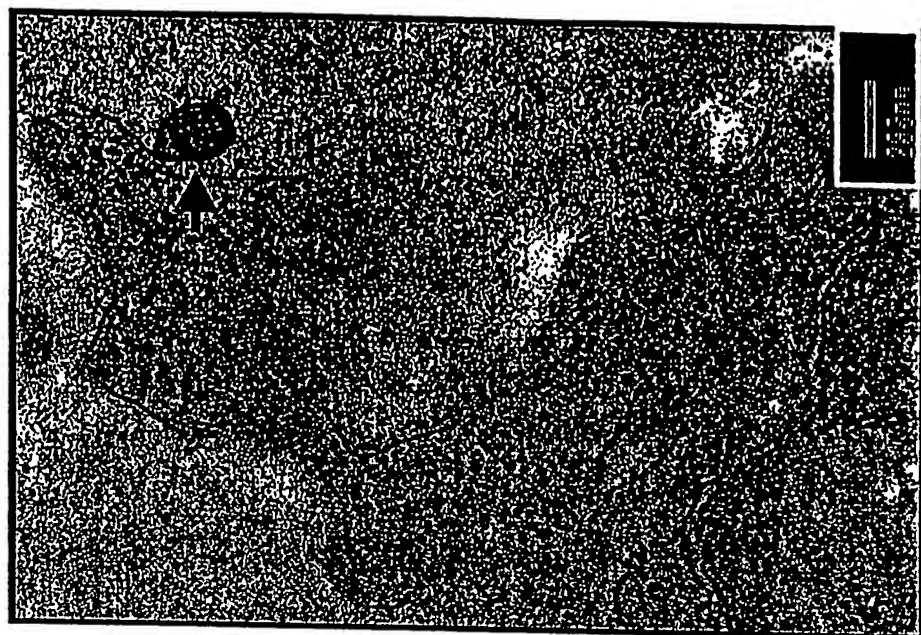


Taken by Dr. A. Nanci, U of Montreal

Electron microscopy of Activated PBLCs; Detection of virus like particles



200 nm

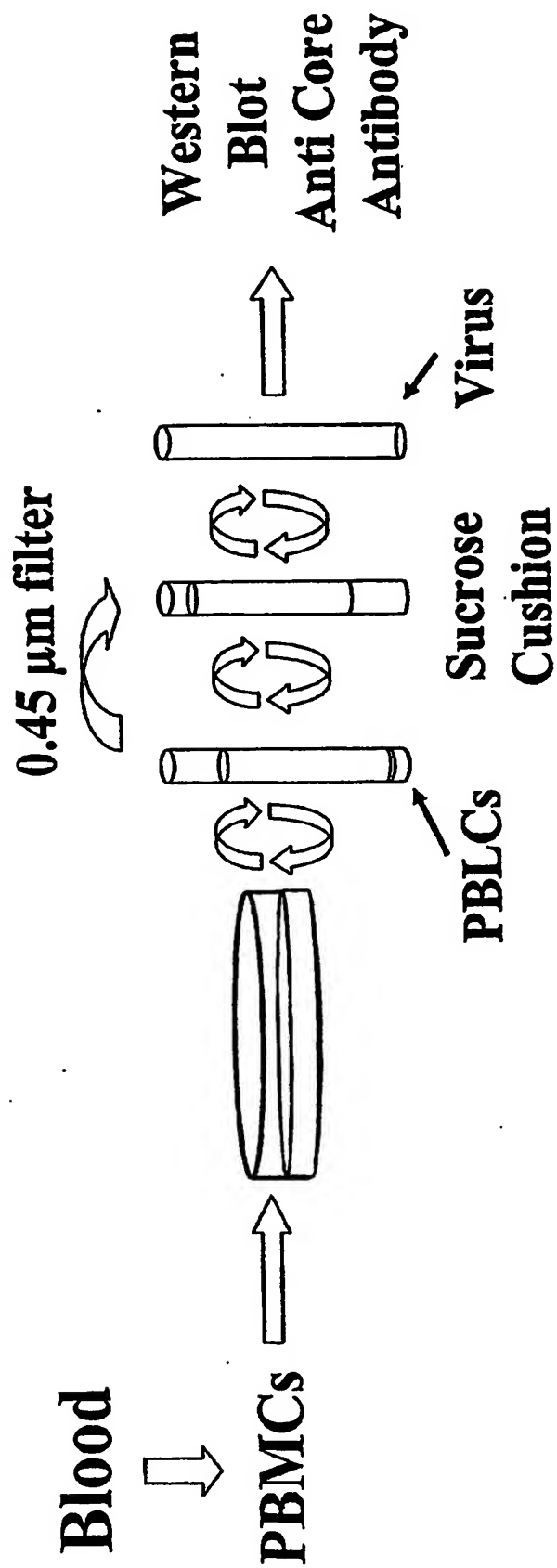


200 nm

FIGURE 11

Taken by Dr. A. Nanci, U of Montreal

Virus partial purification.



Detection of HCV Core protein in supernatant of treated PBMC from an HCV (+) patient.

[Maine biotechnology anti-Core monoclonal antibody]

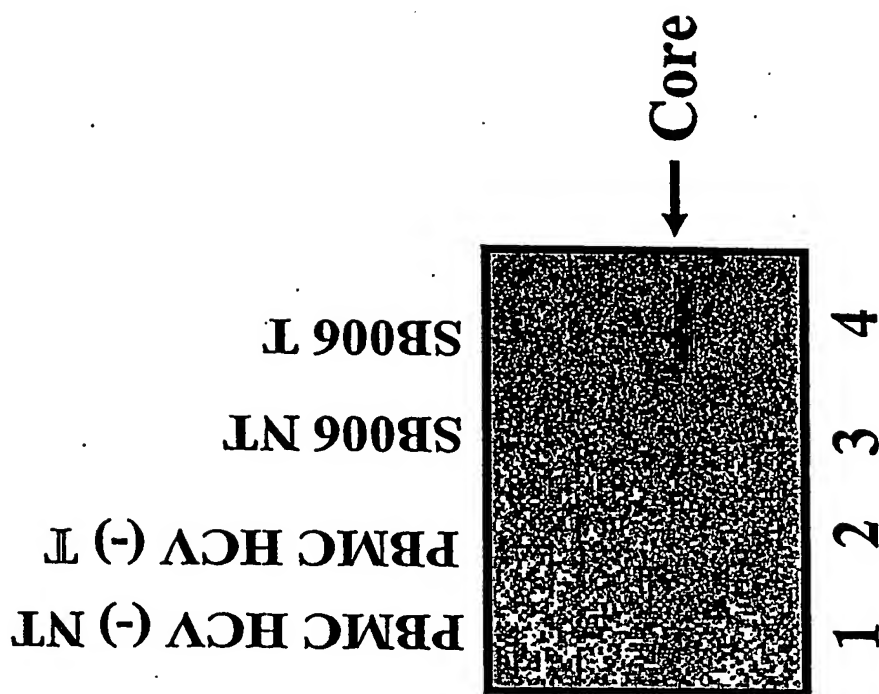


FIGURE 13

RNA Quantification I (virus copies/ng total RNA)

FIGURE 14

Patient
After 4 days

HCV RNA
In PBMC

Detection of
Core (wb) in
supernatant

SB004 NT

2x10³

No

SB004 T

2x10³

Yes

SB006 NT

1.8 x10³

No

SB006 T

2x10²

Yes

After 20 days

SB004

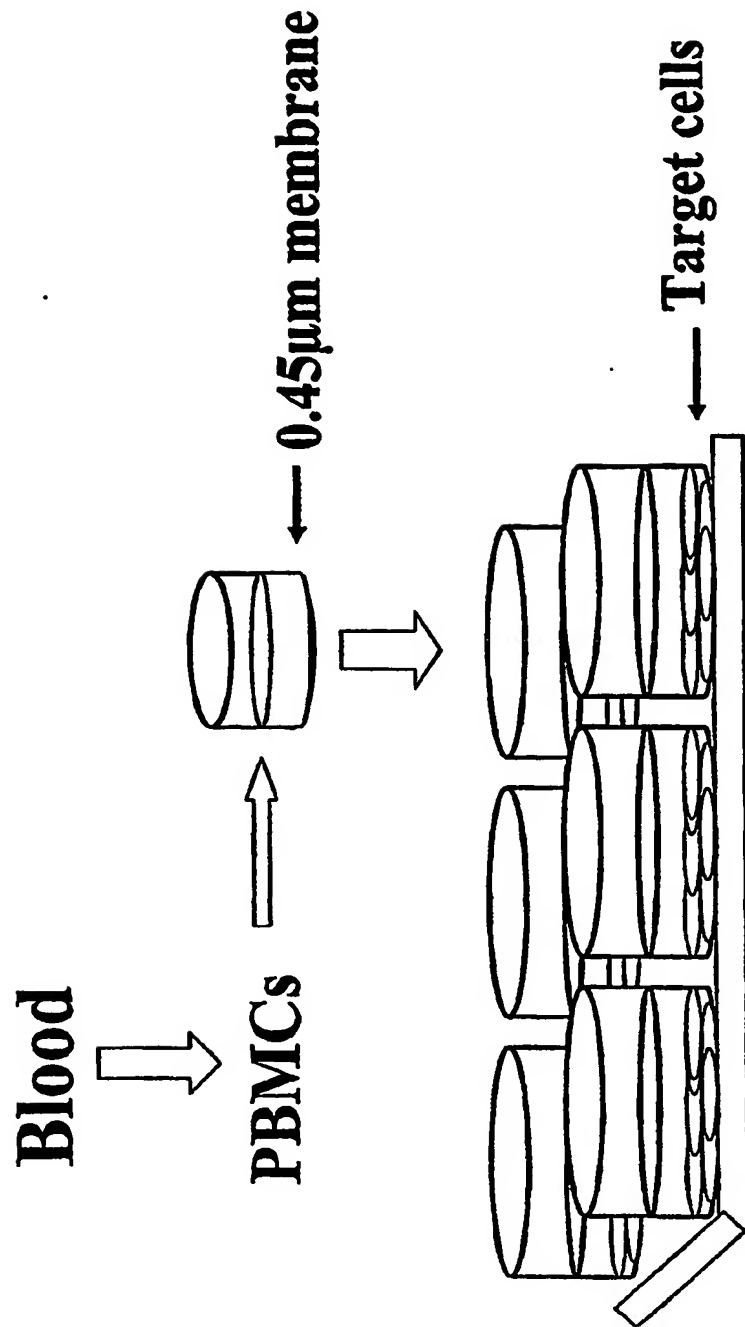
0.00

SB006

0.00

Infection assay; co-culture.

FIGURE 15



Infection of MT-4 cells

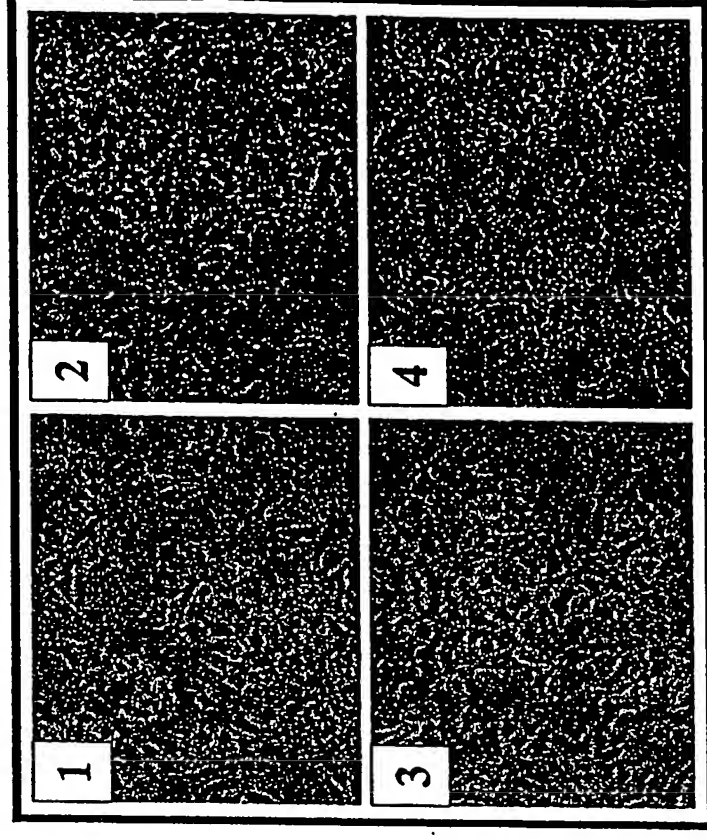
RNA Quantification II (virus copies/ng total RNA)

Patient	HCV RNA In PBMC	Detection of Core (wb) in supernatant	HCV RNA In MT-4
<u>After 10 days</u>			
SB001 NT	13	No	0.00
SB001 T	12	Yes	1600
<u>After 20 days</u>			
SB001	0.00		0.00
SB001	0.00		0.00

FIGURE 16

Co-culture of Huh-7 and HCV (-) PBMCs.

- 1- Huh-7
- 2- Huh-7 + PBMCs HCV (-) NT
- 3- Huh-7 + Treatment
- 4- Huh-7 + PBMCs HCV (-) T



Co-culture of Huh-7 and HCV (+) PBMCs (+) PBMCs (SB00)

1. Huh-7
- 2-3. Huh-7 + PBMCs HCV (+) NT
4. Huh-7 + Treatment
- 5-6. Huh-7 + PBMCs HCV (+) T

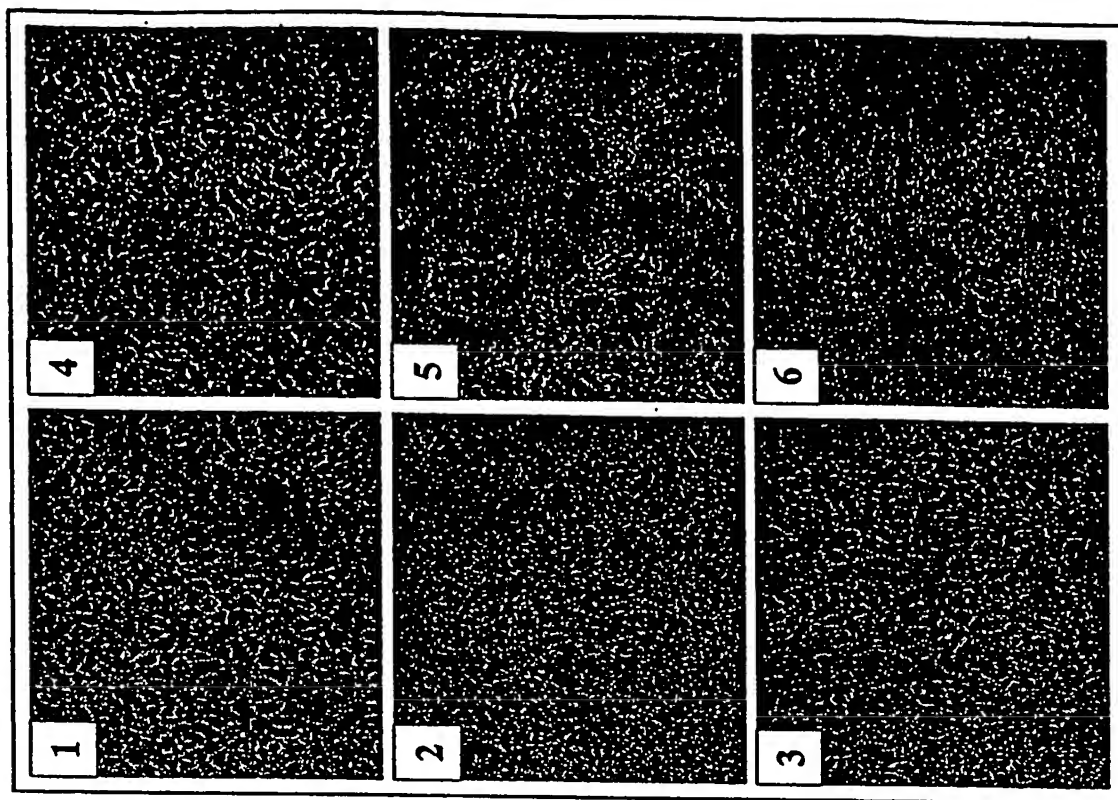
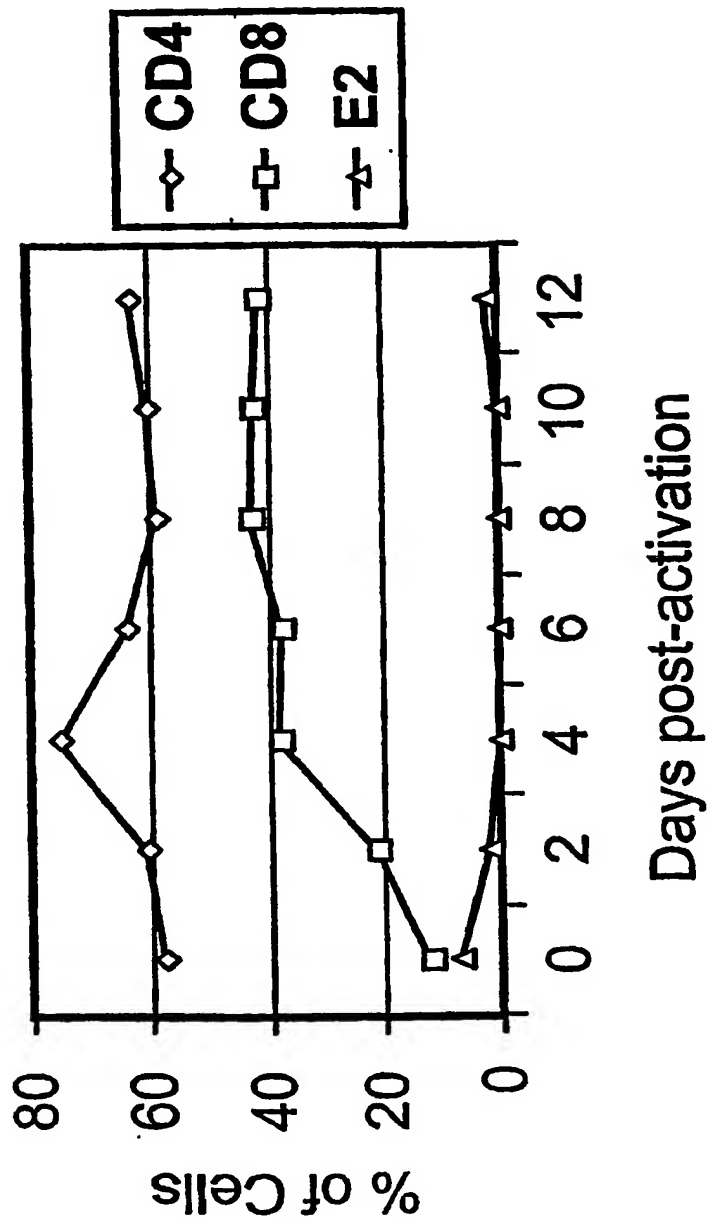


FIGURE 10

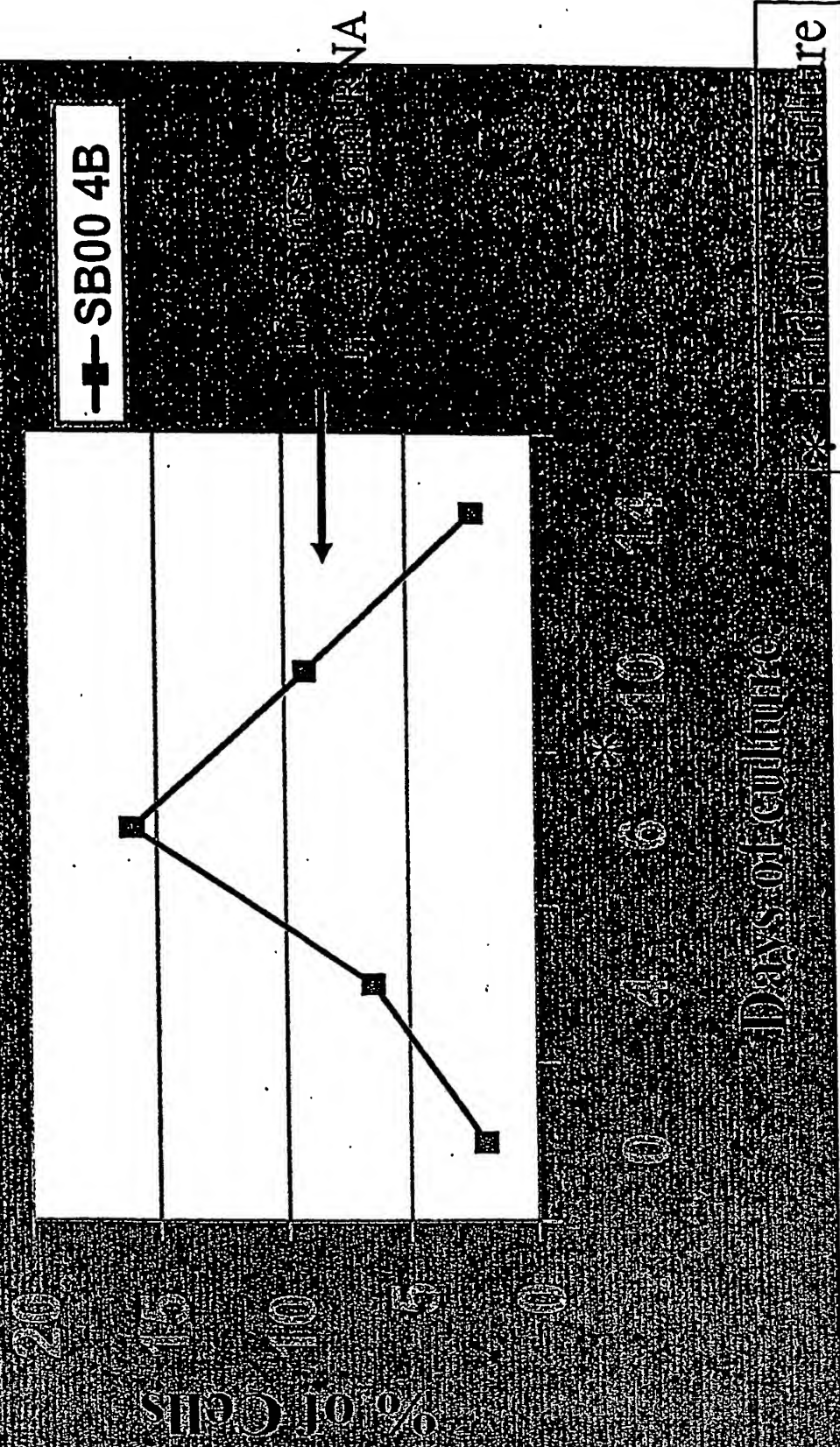
**PHA Activation of PBMCs from patient SB004;
HCV is not in T cells**

FIGURE 19



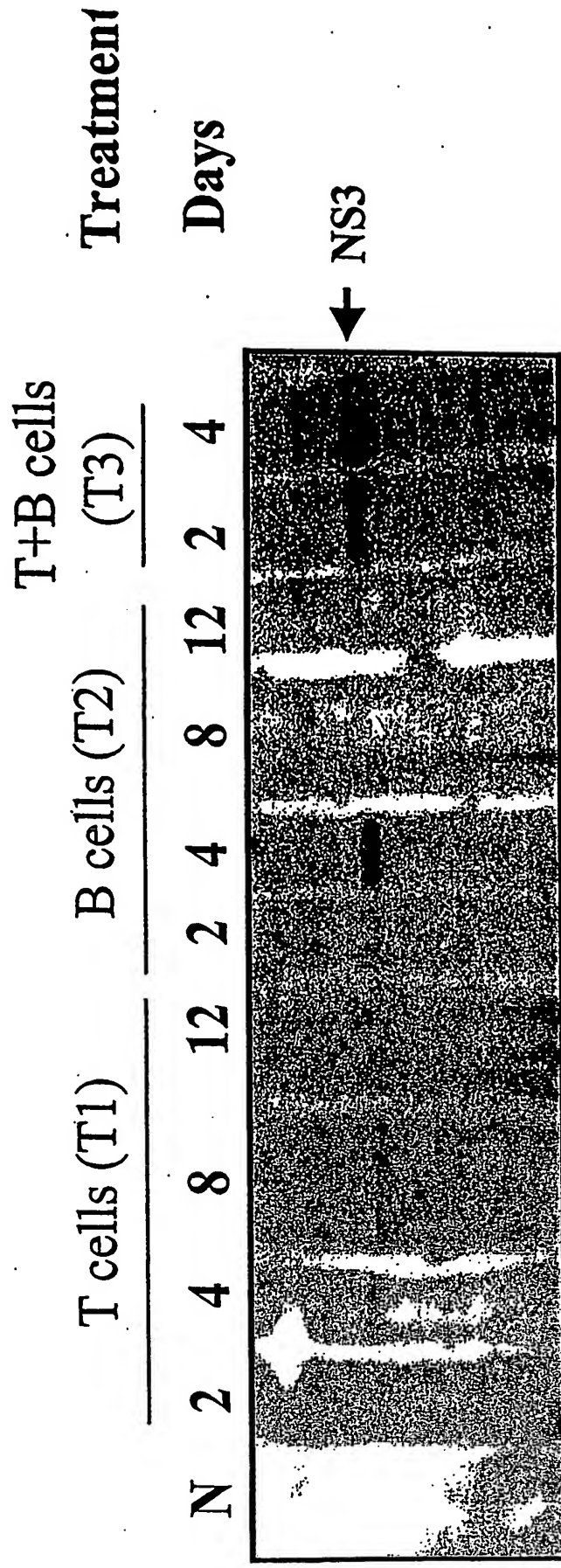
Detection of HCV (E2) on Daudi cells

Upon co-cultivation with infected PBMCs



* End of culture

Comparison of different activation treatments;
PBMCs from donor MLL-010



Viral RNA in cell supernatant (Real time RT-PCR).

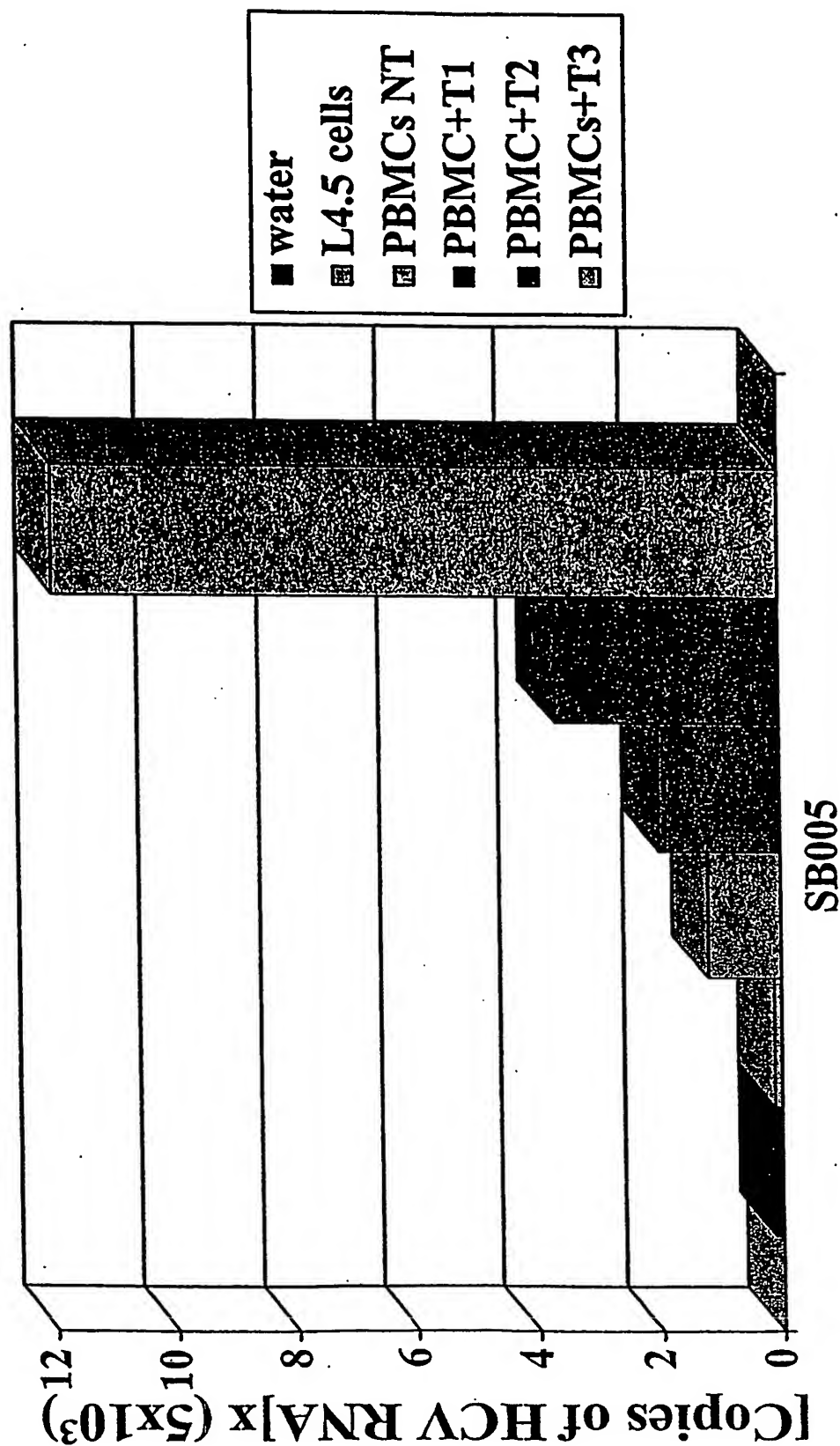


Fig. 23c

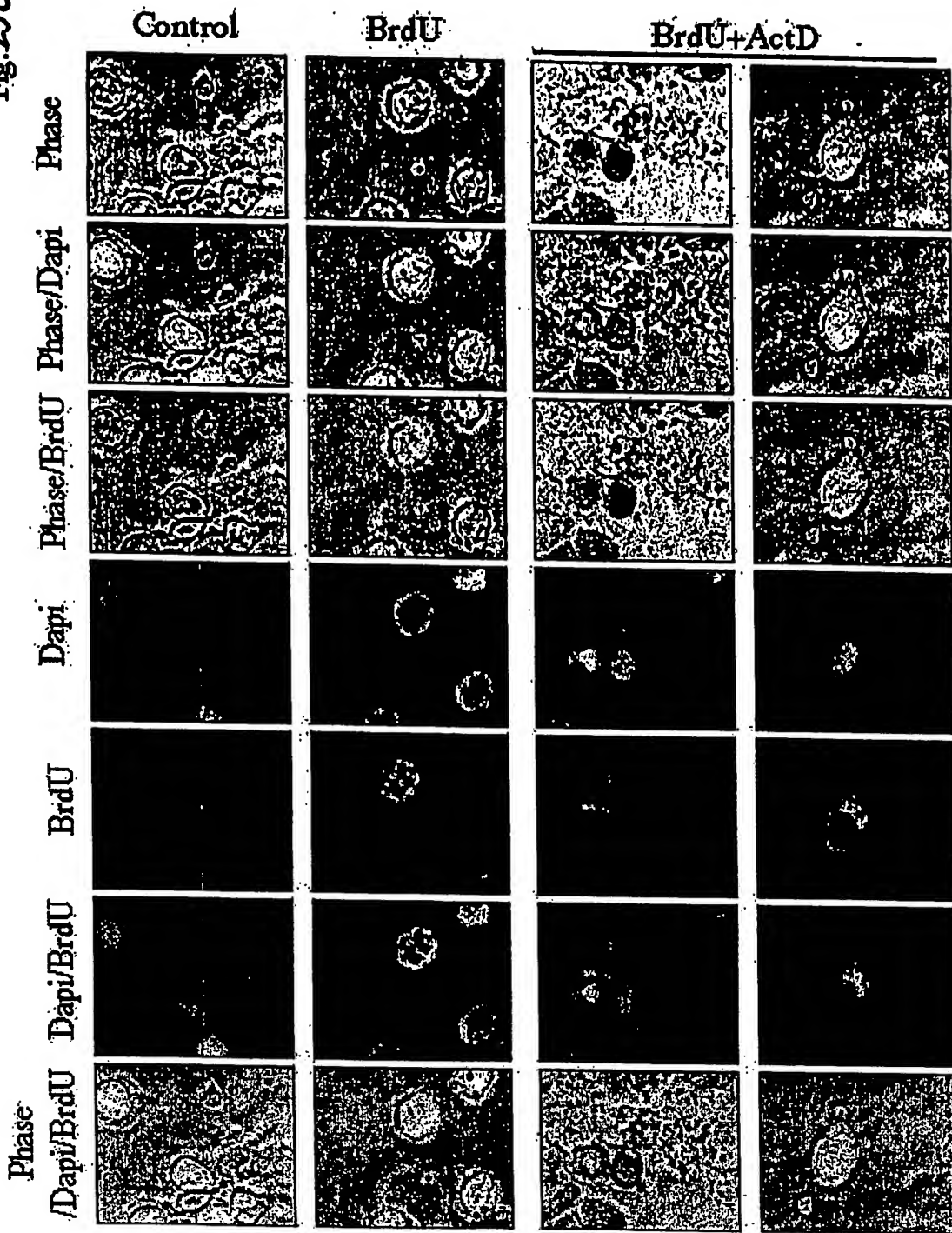


Fig.29A

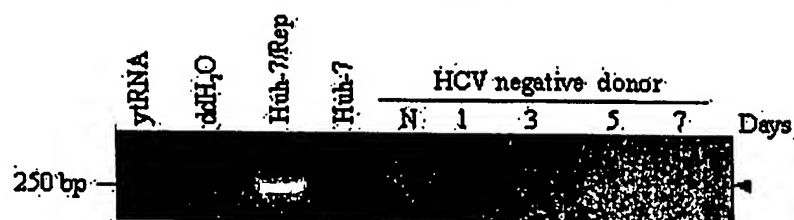
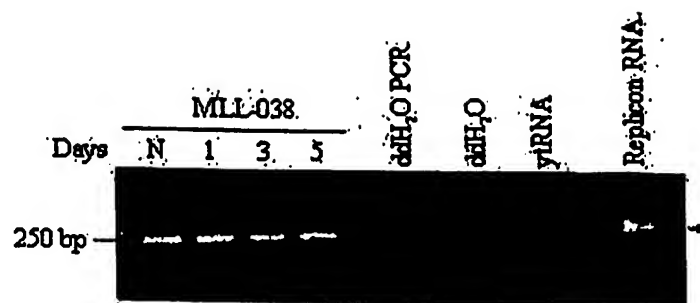


Fig.23B

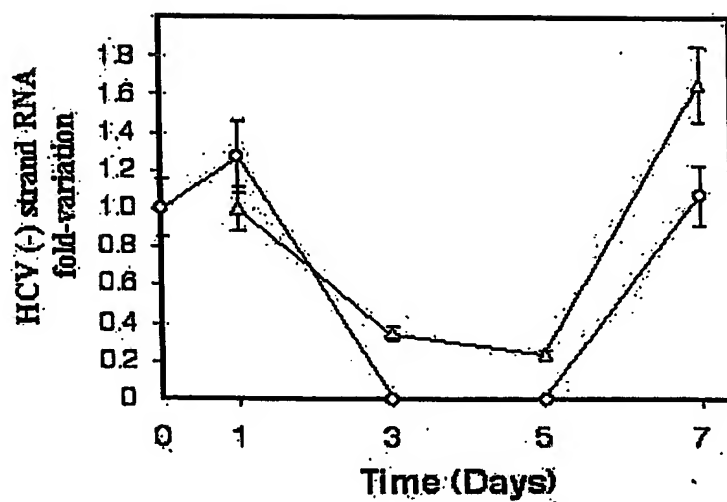


Fig. 23 D

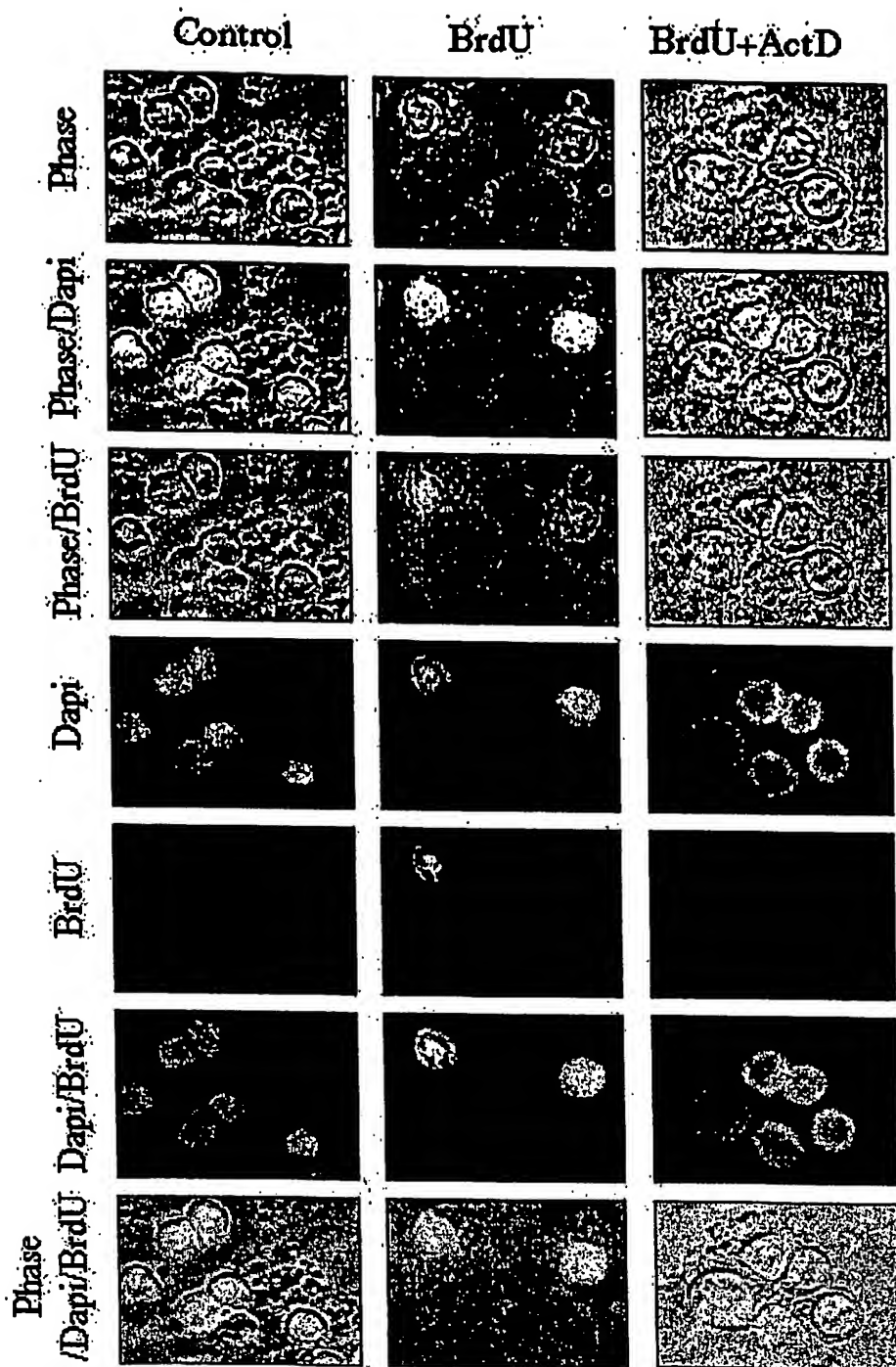


Fig 24A

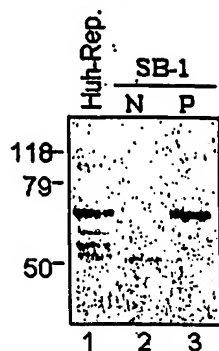


Fig 24B

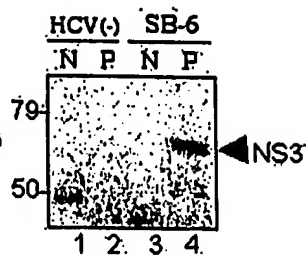


Fig 24C

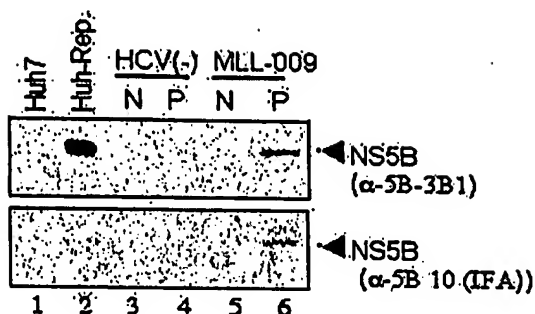


Fig 24D

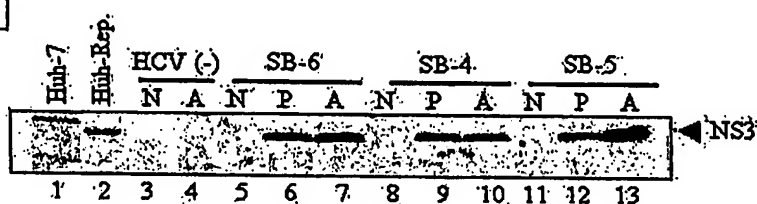


Fig 24E

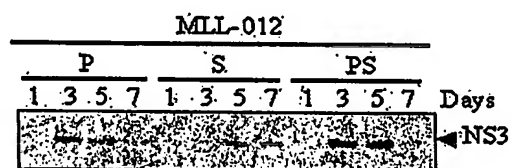


Fig 24F

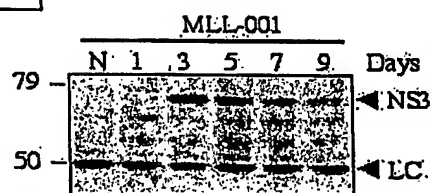


Fig 24H

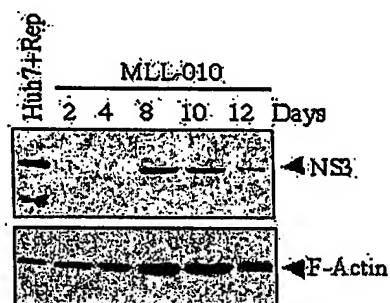


Fig 24G

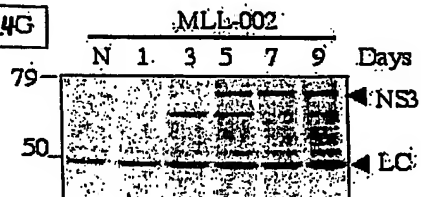


Fig24J

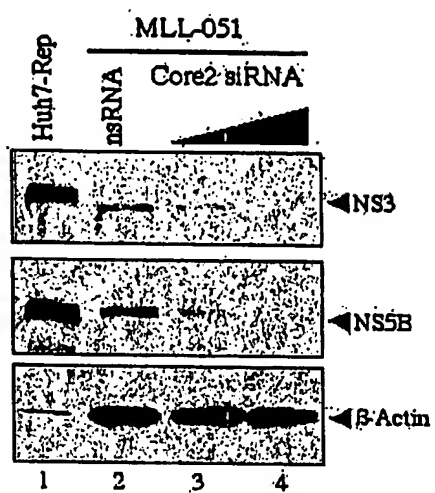


Fig24I

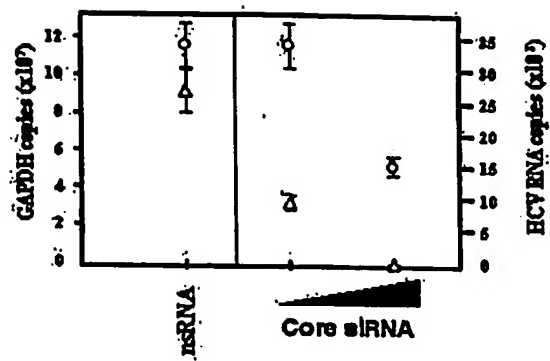


Fig24K

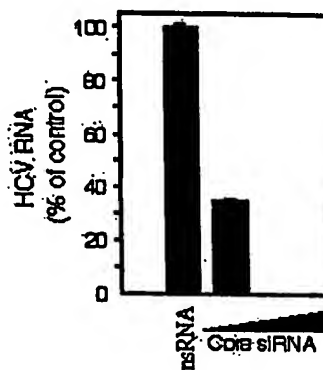


Fig.25

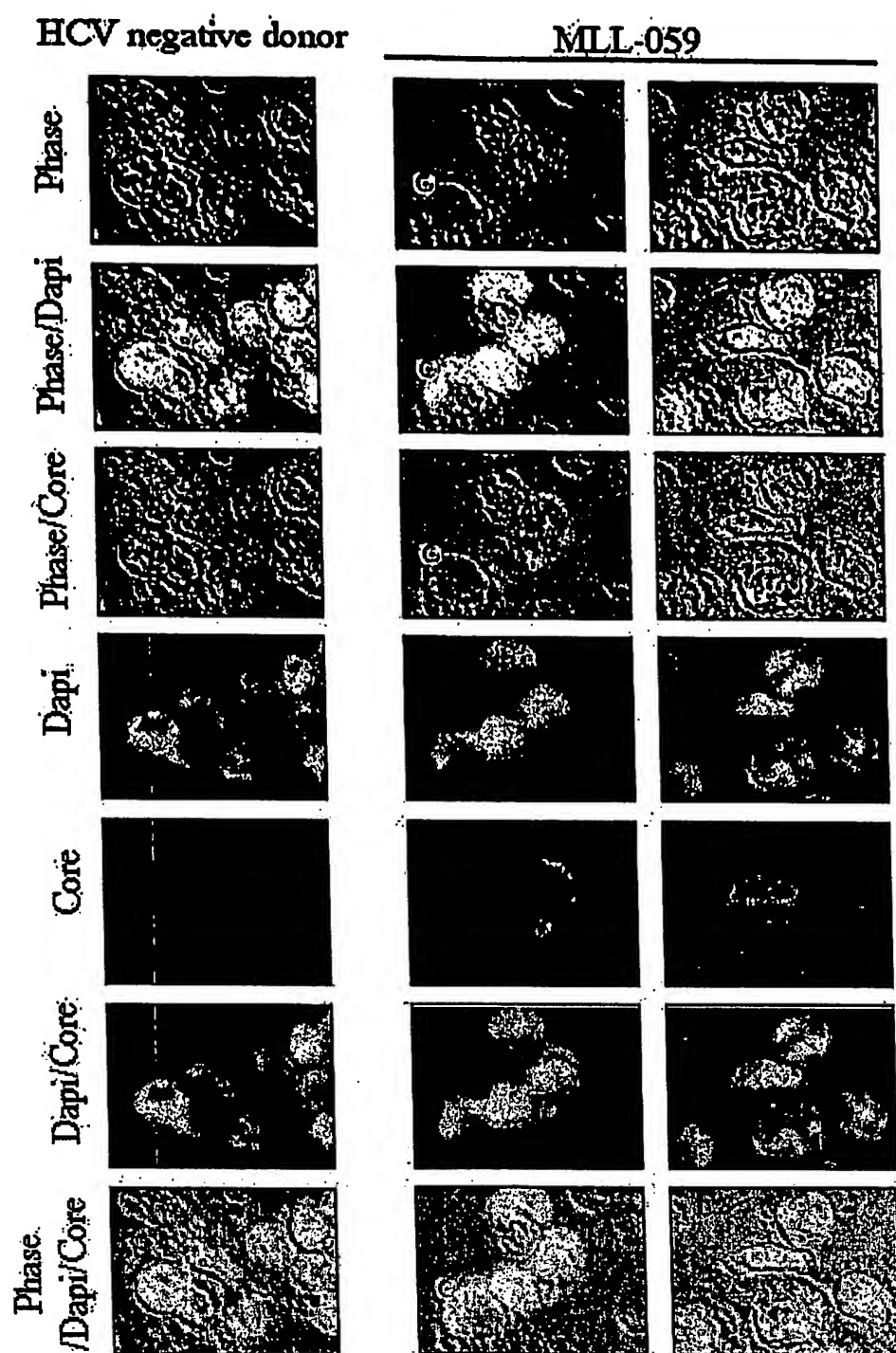


Fig. 26

Fig 26A

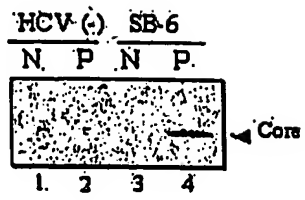


Fig 26B

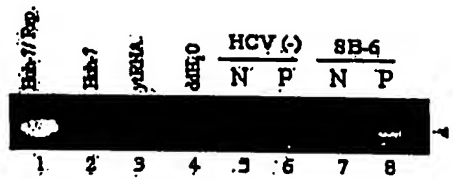


Fig. 26C

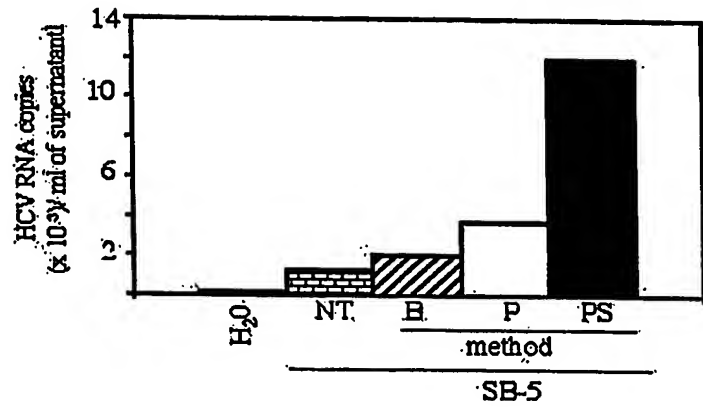


Fig 26D

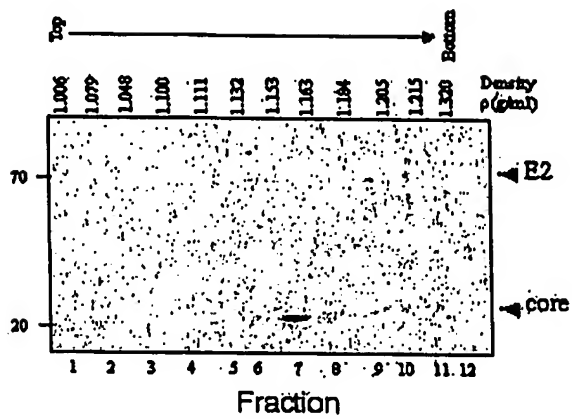


Fig 26E

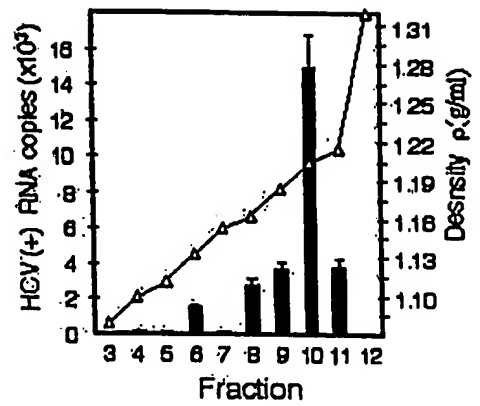


Fig 26F

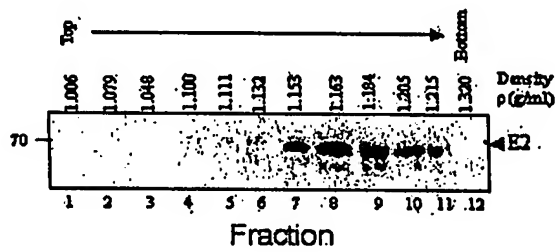


Fig 26G

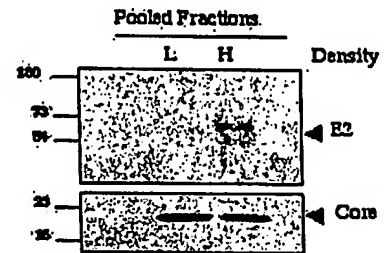


Fig. 26

Fig 26H

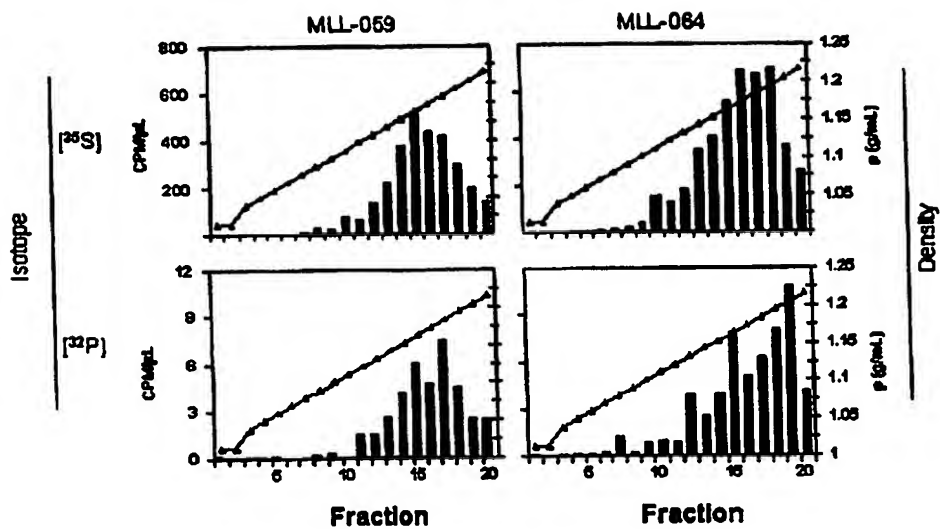


Fig. 27

Fig. 27A

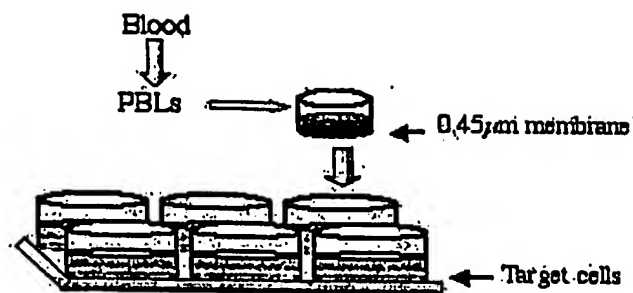


Fig. 27B

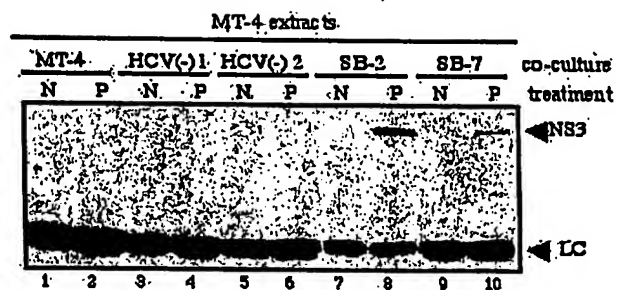
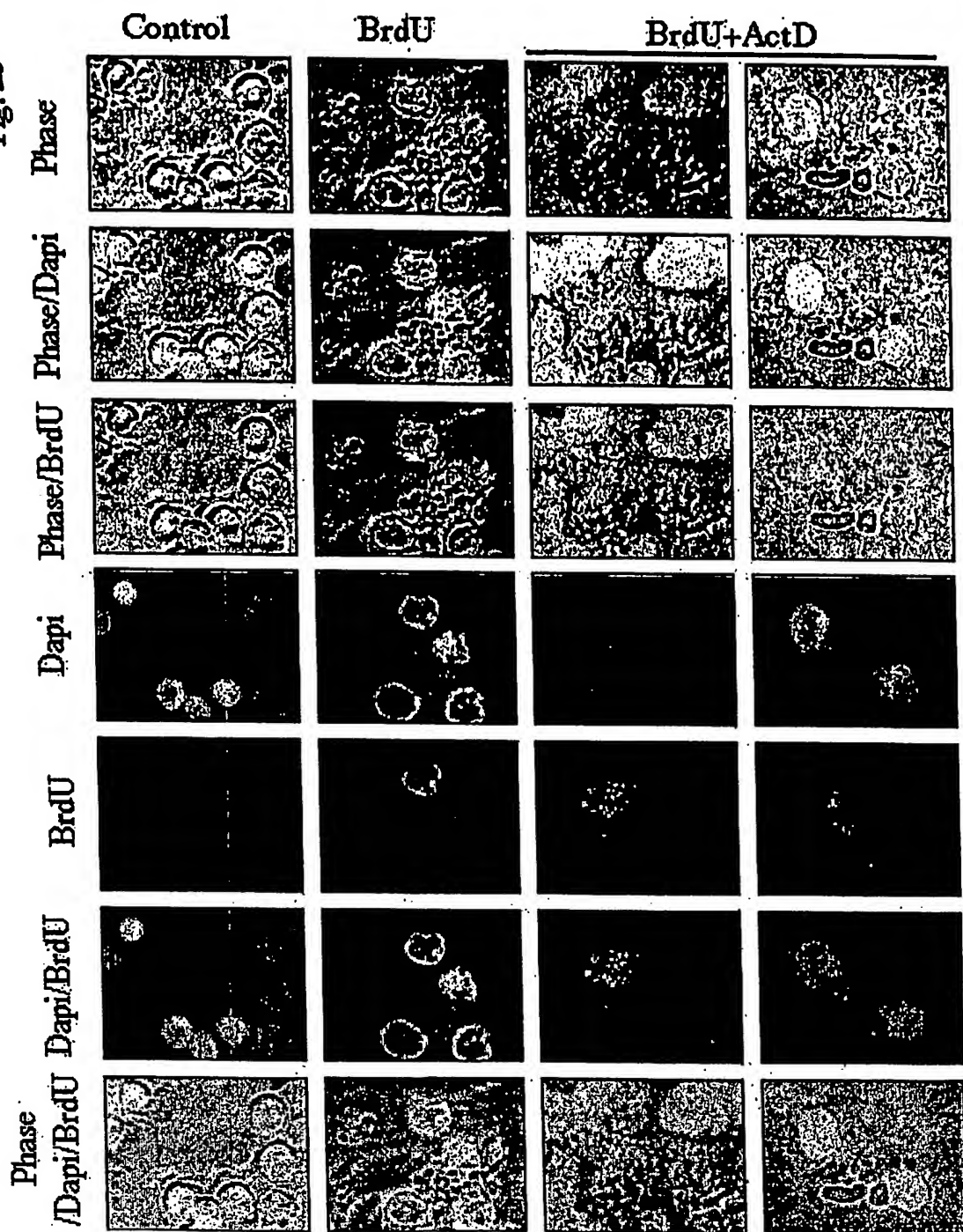
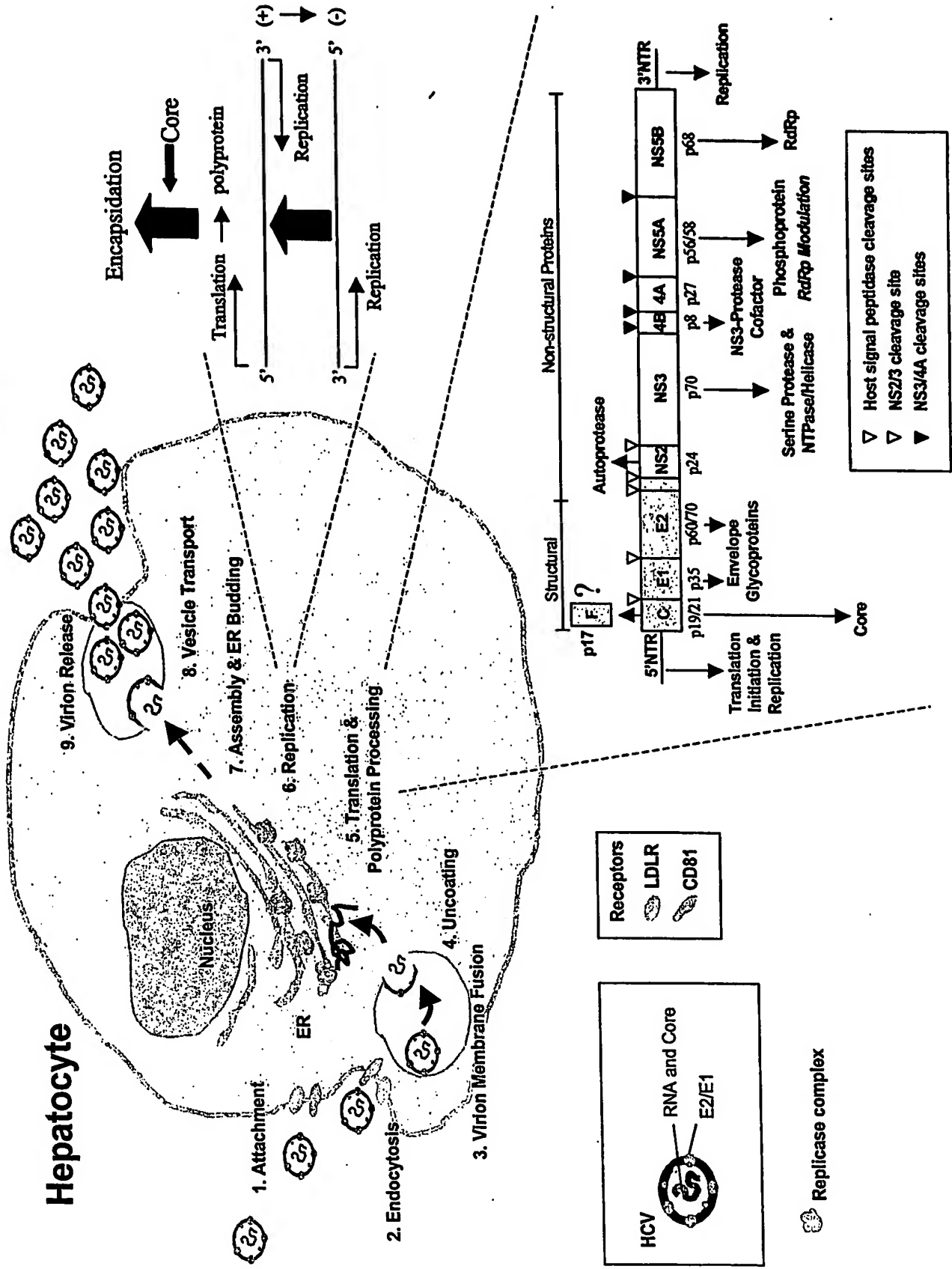


Fig. 28



HCV Replication Cycle



Protocol for quantitative LightCycler analysis of viral Hepatitis C RNA levels.

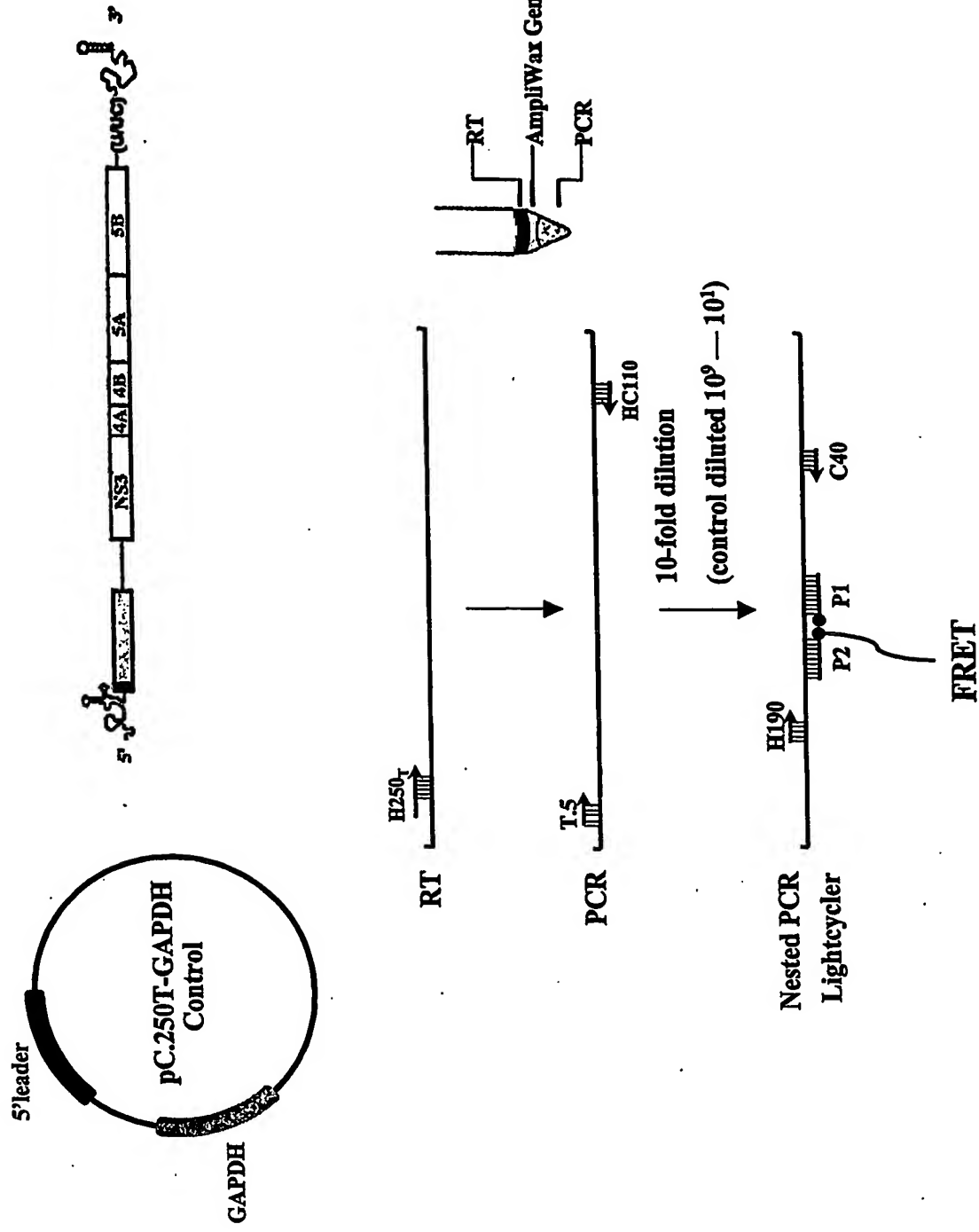


Figure 31

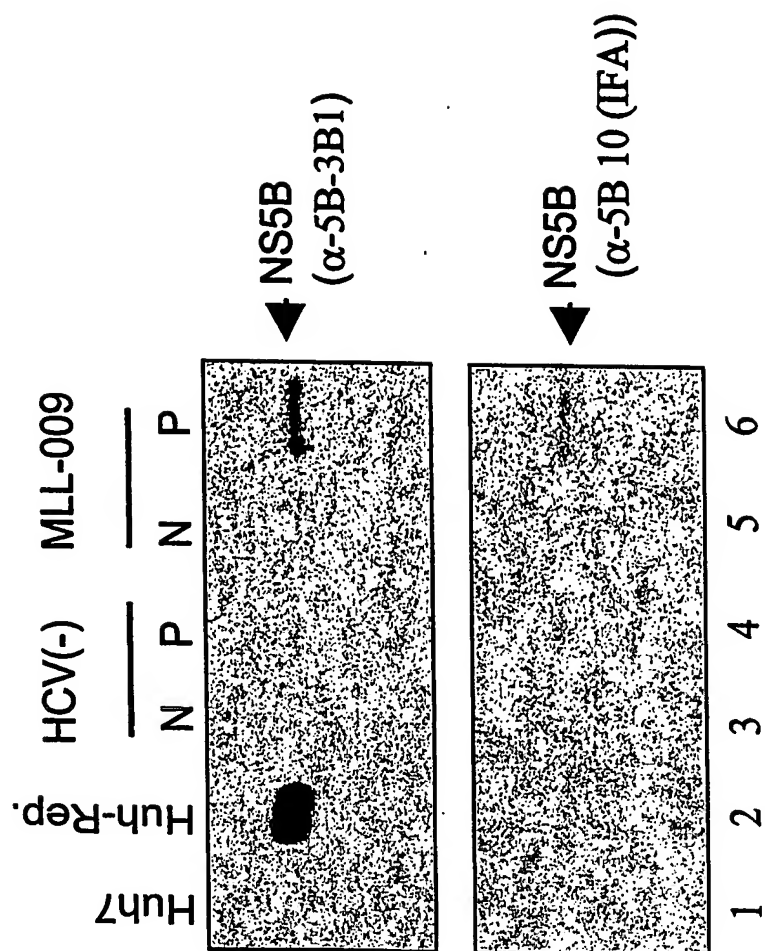
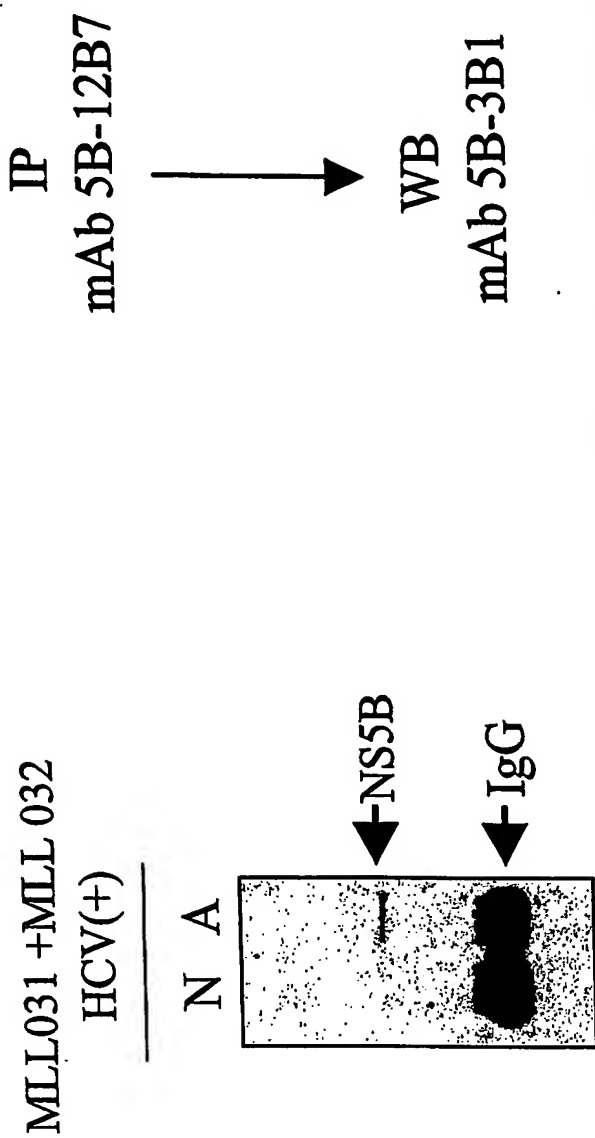
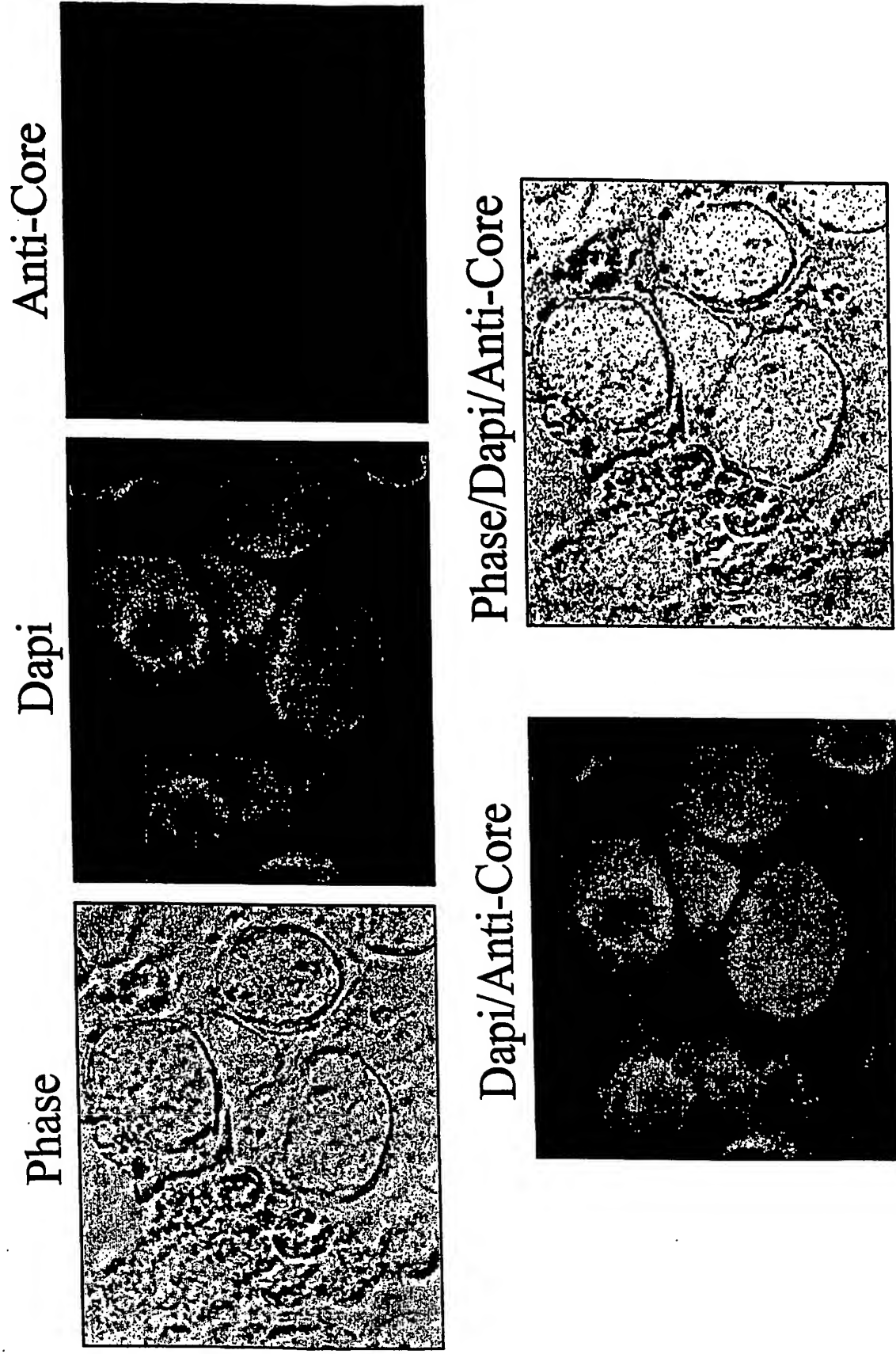


FIGURE 32

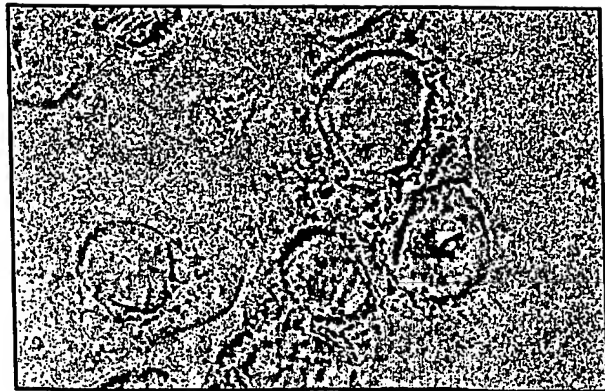


[D. Moradpour & R. Bartenschlager
J. Biol. Chem., 2002, 277, 593-601]

Figure 33



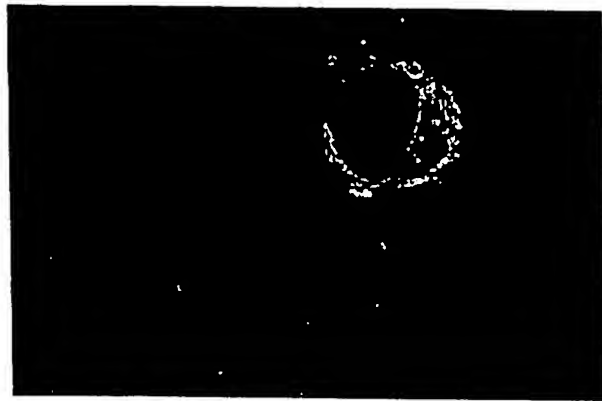
Phase



Dapi



Anti-Core



Dapi/Anti-Core

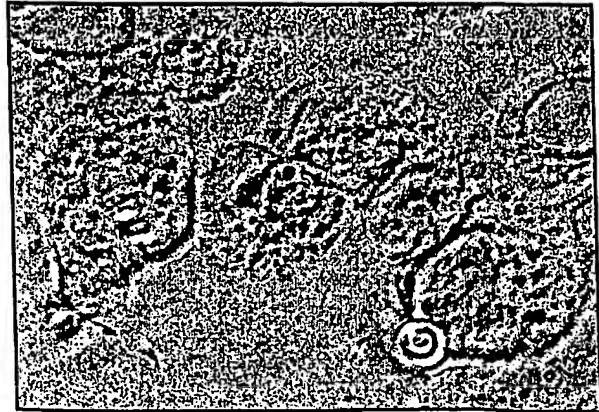


Phase/Dapi/Anti-Core

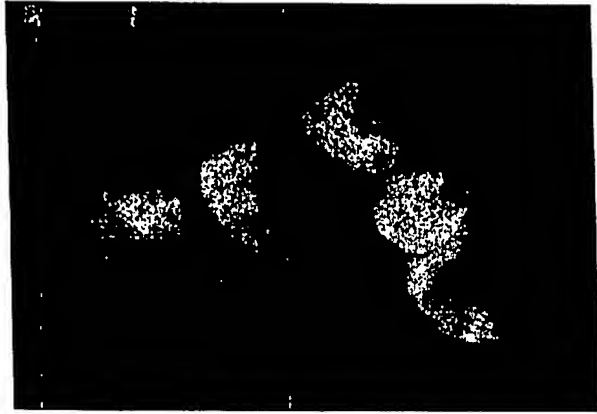


Figure 34

Phase



Dapi



Anti-Core



Dapi/Anti-Core



Phase/Dapi/Anti-Core

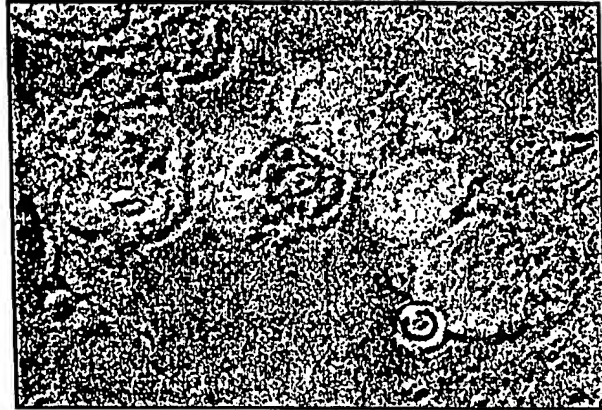


Figure 35

Figure 36

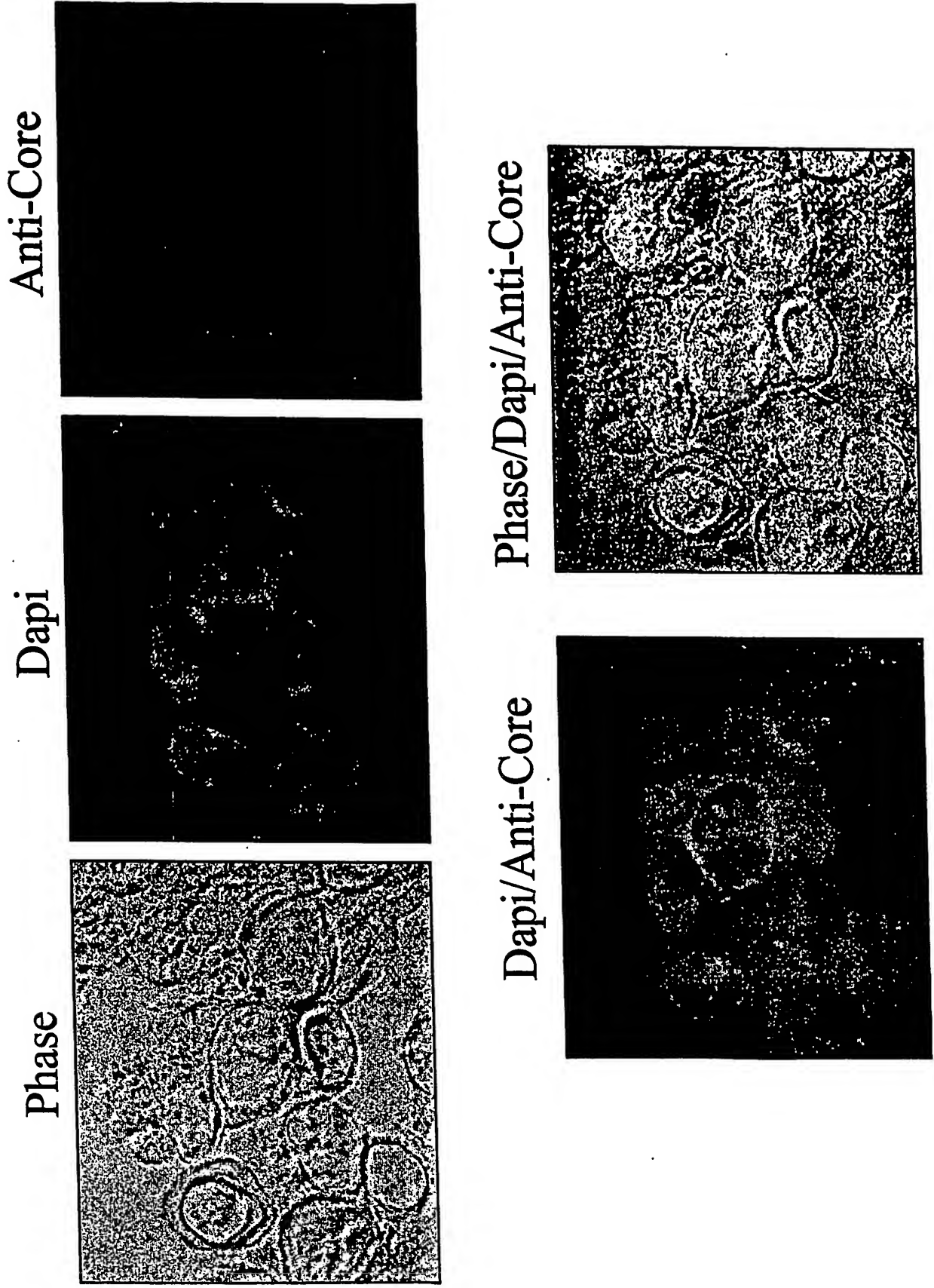


Figure 37

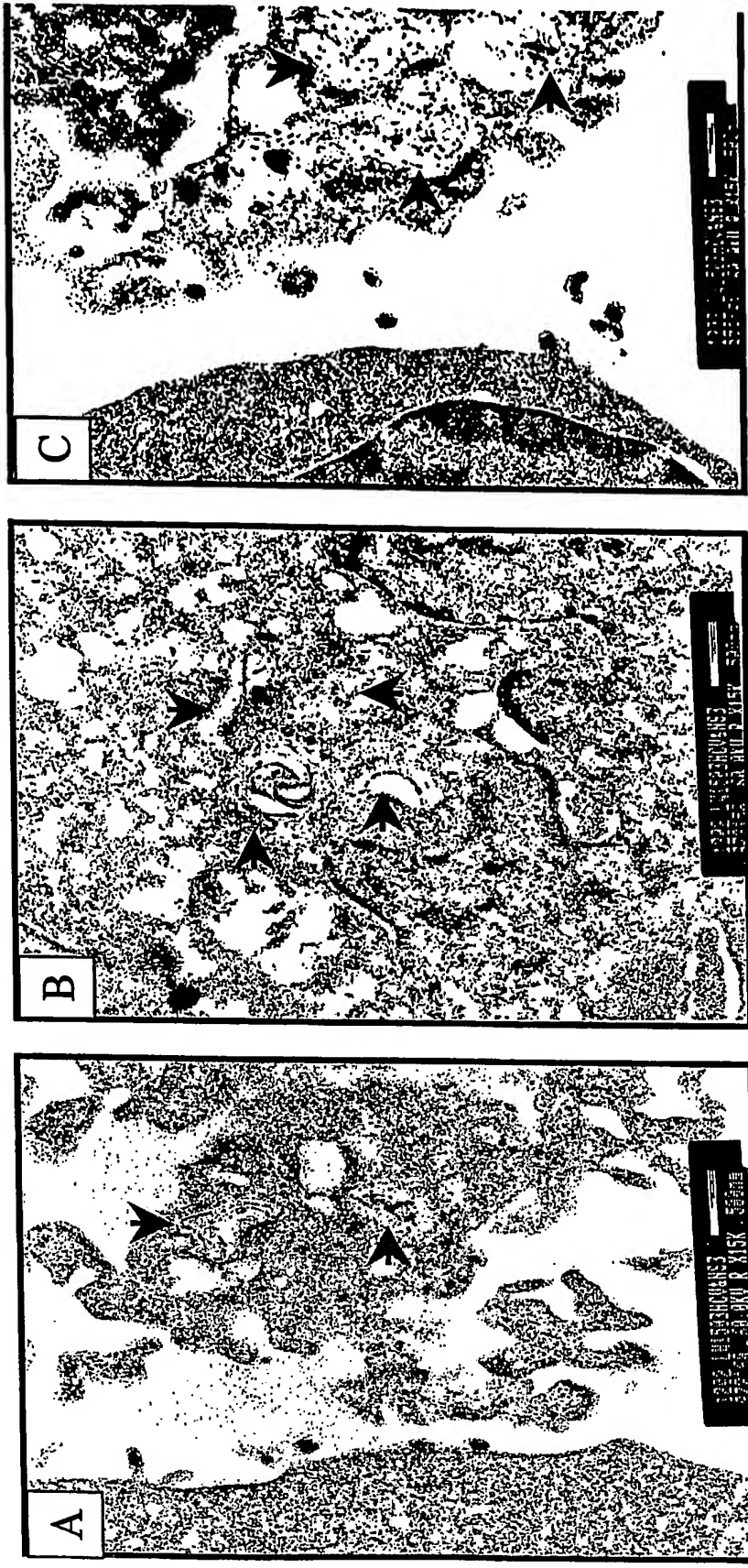


Figure 38

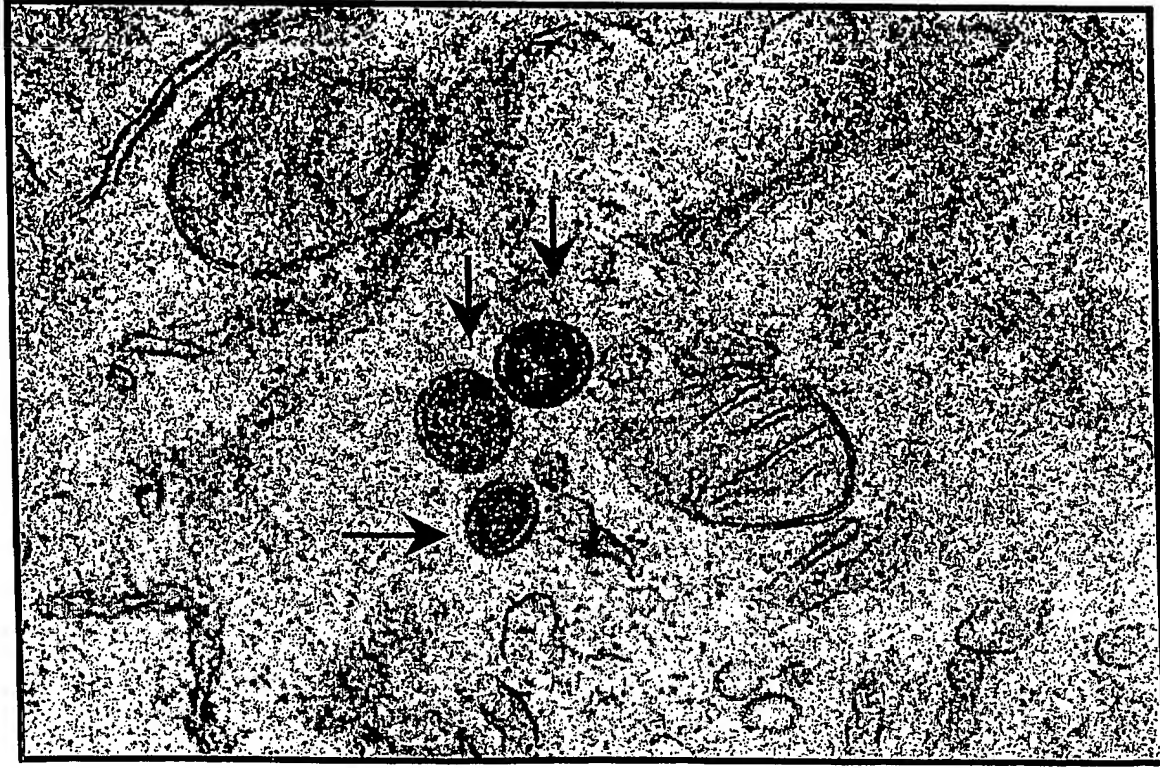
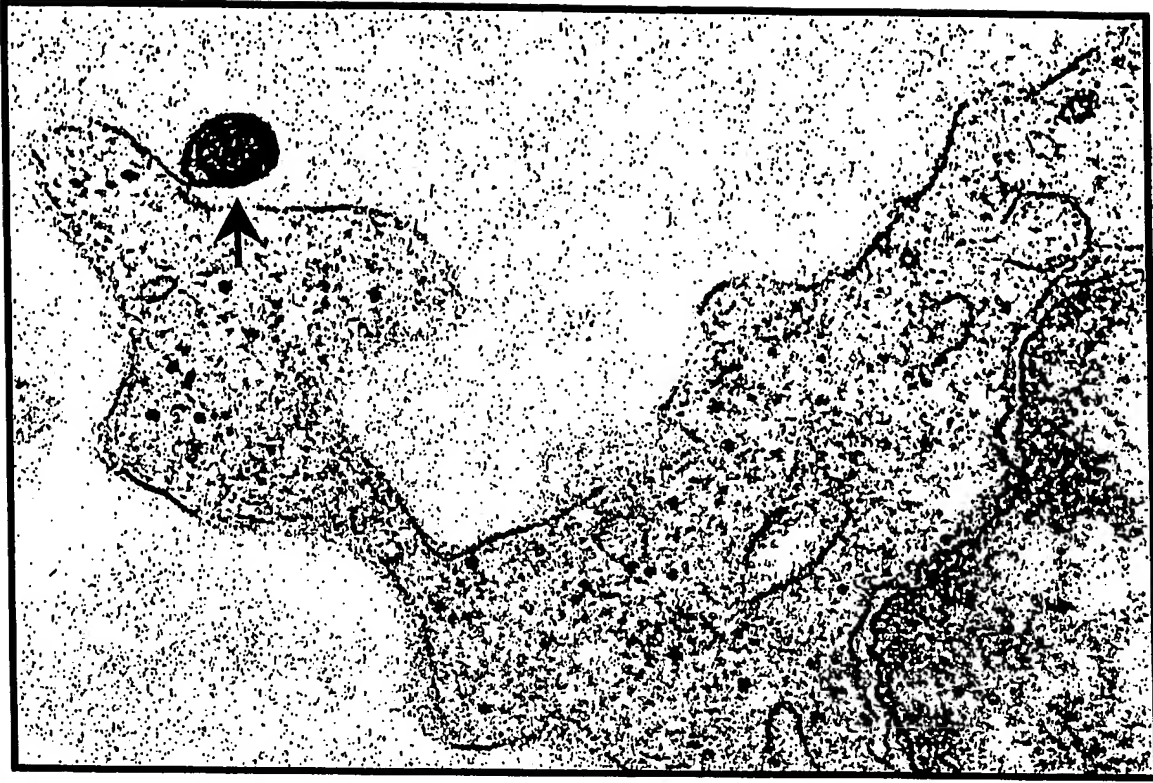


Figure 39: Virus partial purification.

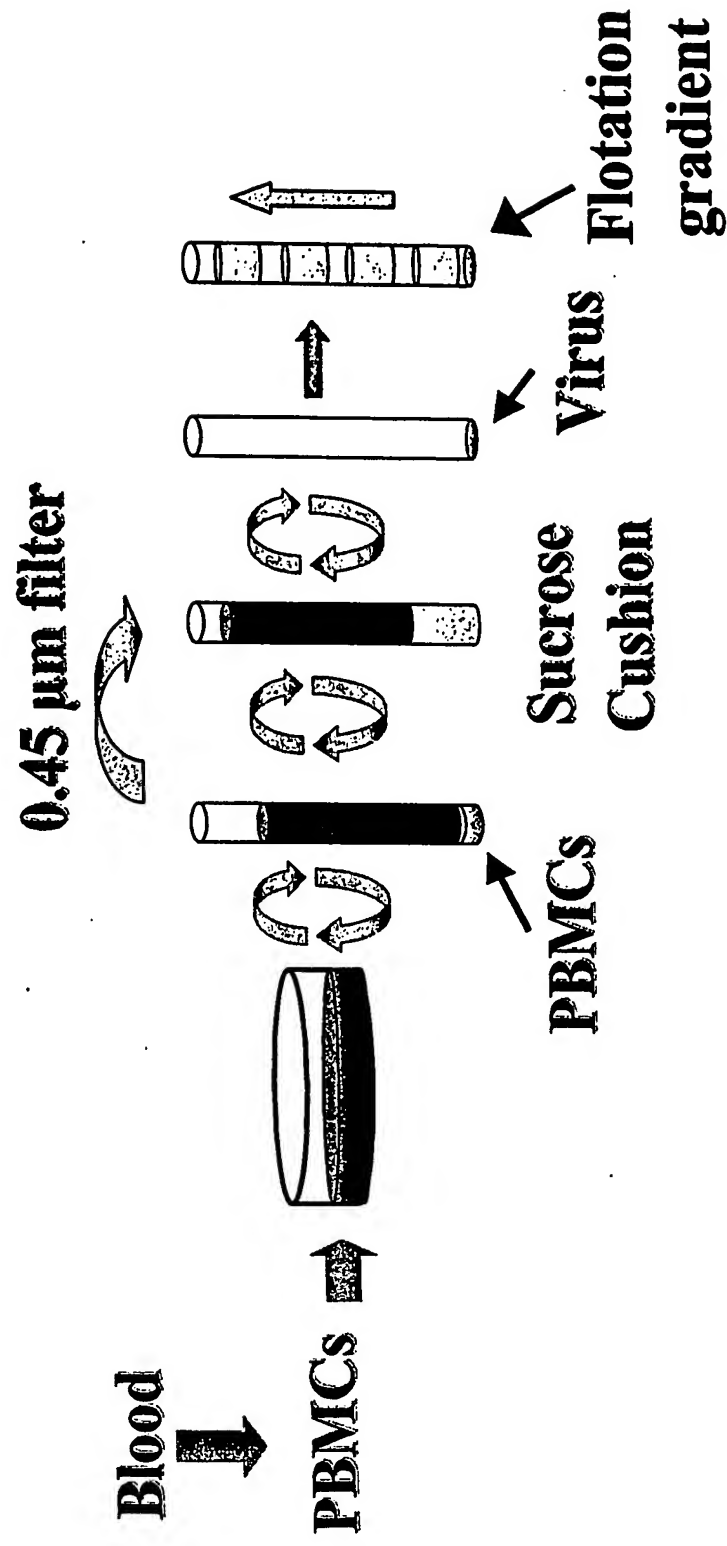


Figure 40

Density Range (g/ml)	Source	Reference
1.15-1.20	HCV-LP in VSV vector	J.Virol (2002) 76, 12325.
1.14-1.18	HCV-LP in insect cells	J. Virol (1998) 72, 3827.
1.12-1.17	Plasma chimps	J. Gen.Virol (1994) 75, 1755
1.09-1.21	Plasma chimps	J.Med.Virol (1991), 34, 206.
1.13-1.17	Plasma chimps	J.Virol (1993) 67, 1953
1.063-1.21	Serum infected donors	J Med Virol (2002) 68, 335
1.11-1.215	HCV(+) PBMCs	-----

Figure 41

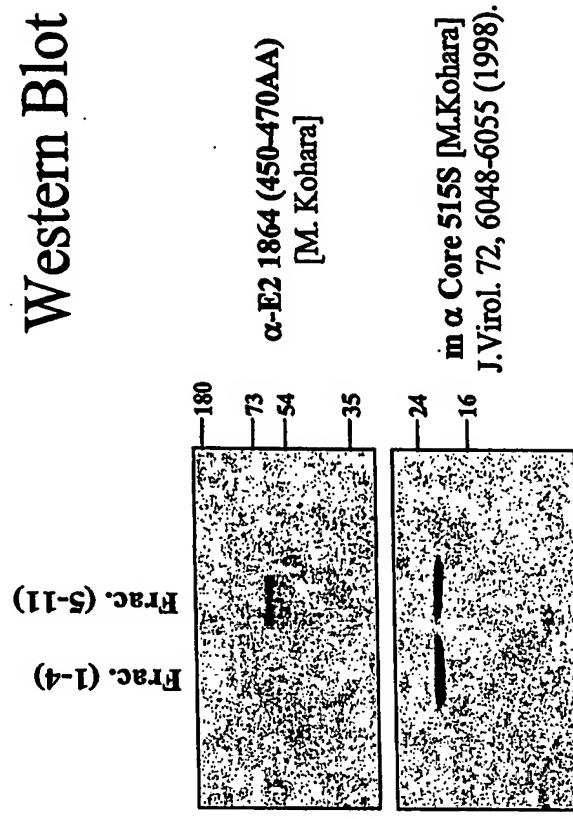


Figure 42

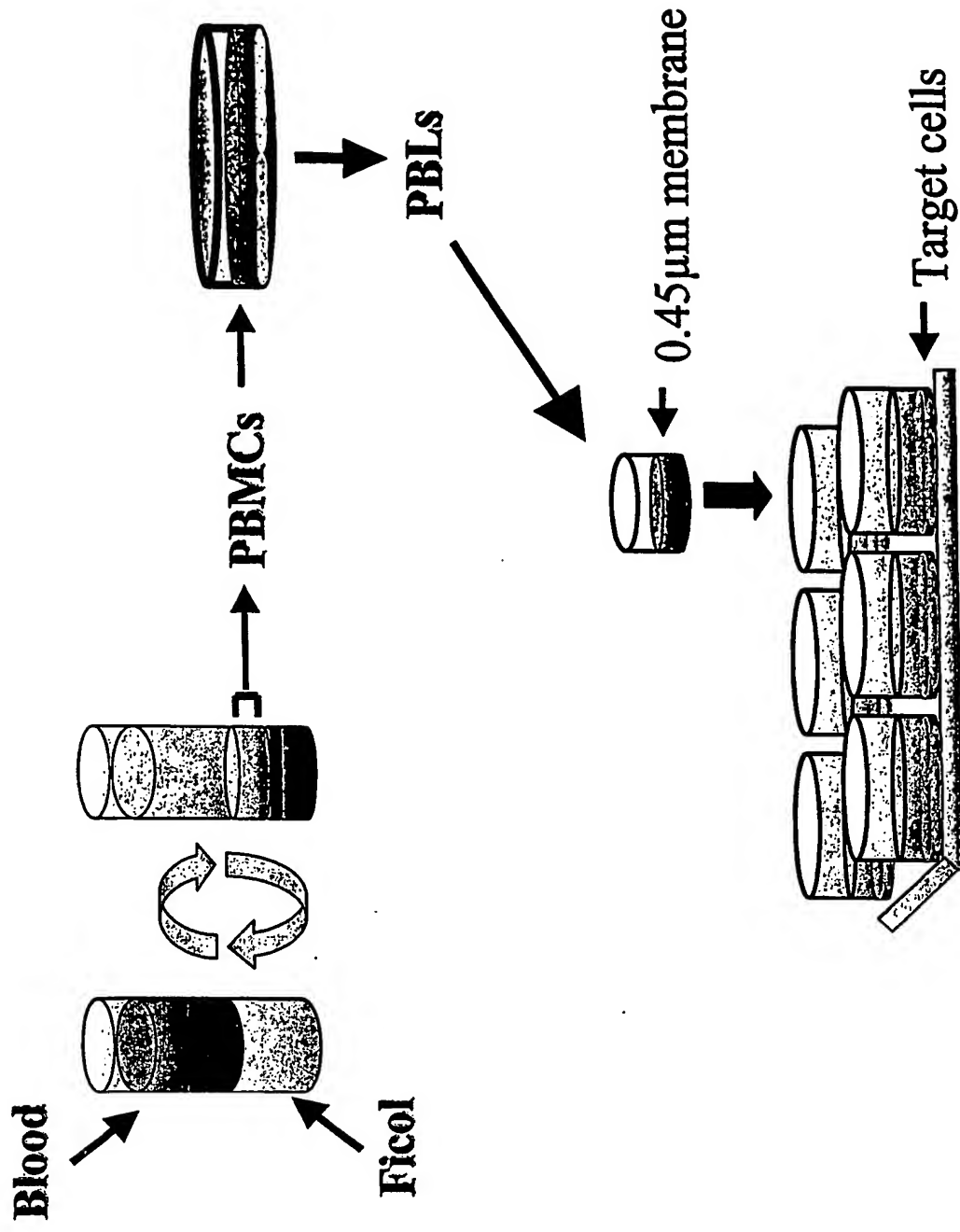


Fig.43

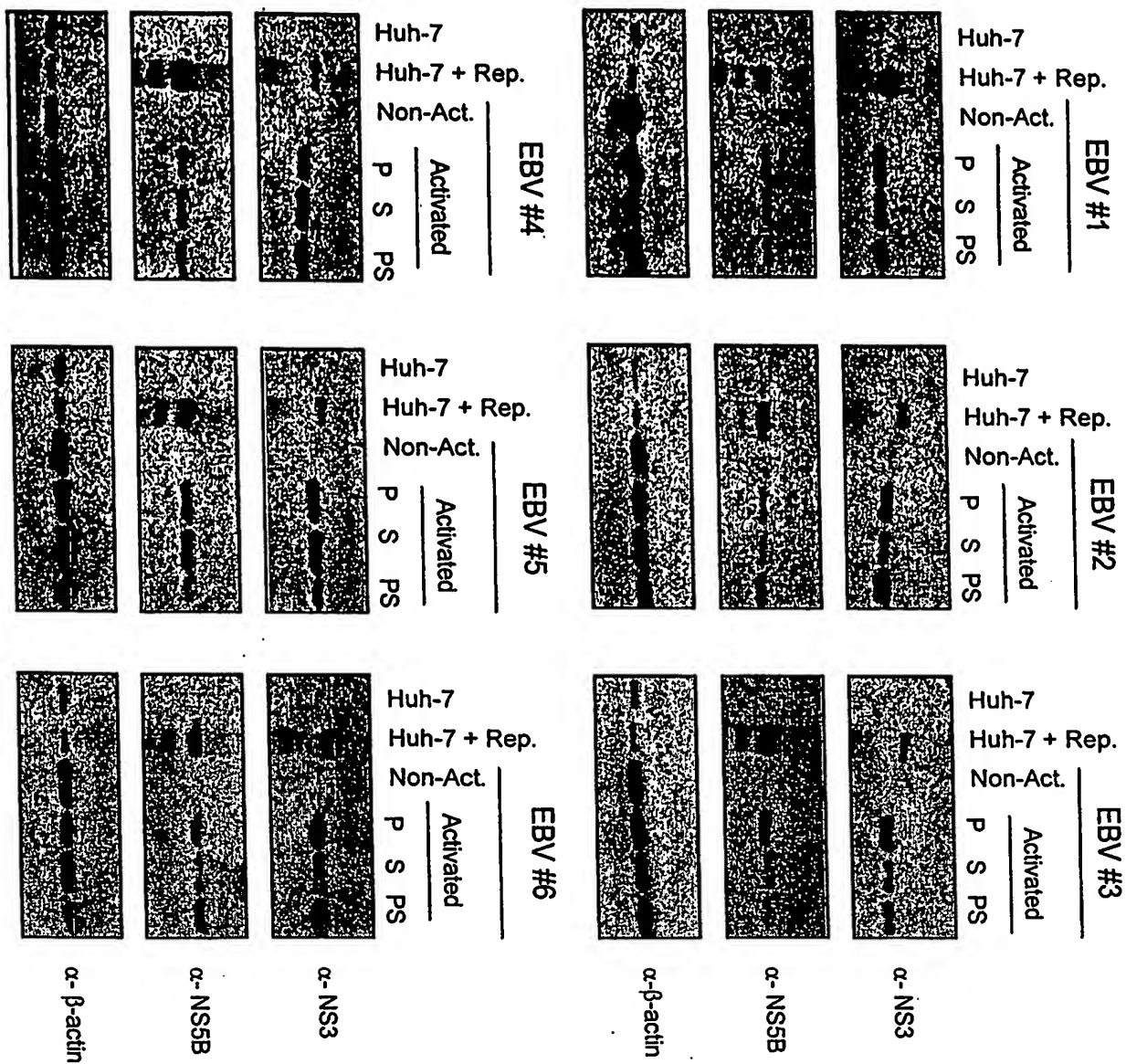
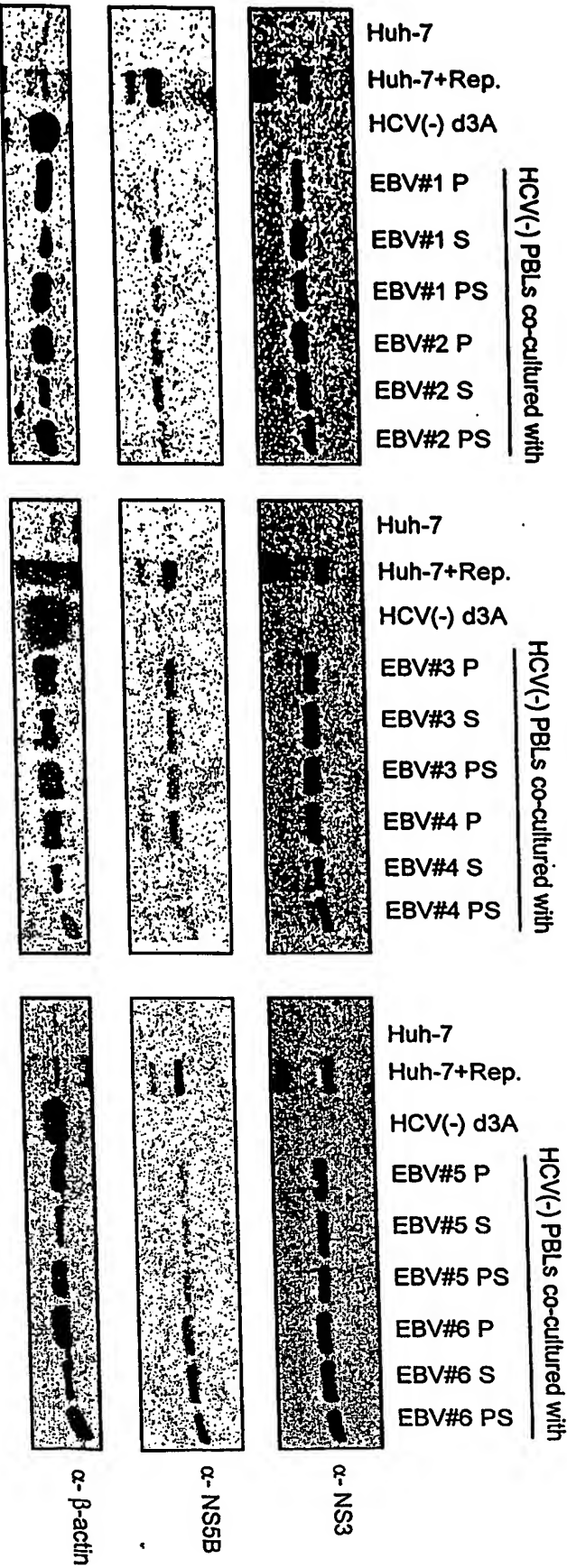


Fig.44



I- Translation Activator.

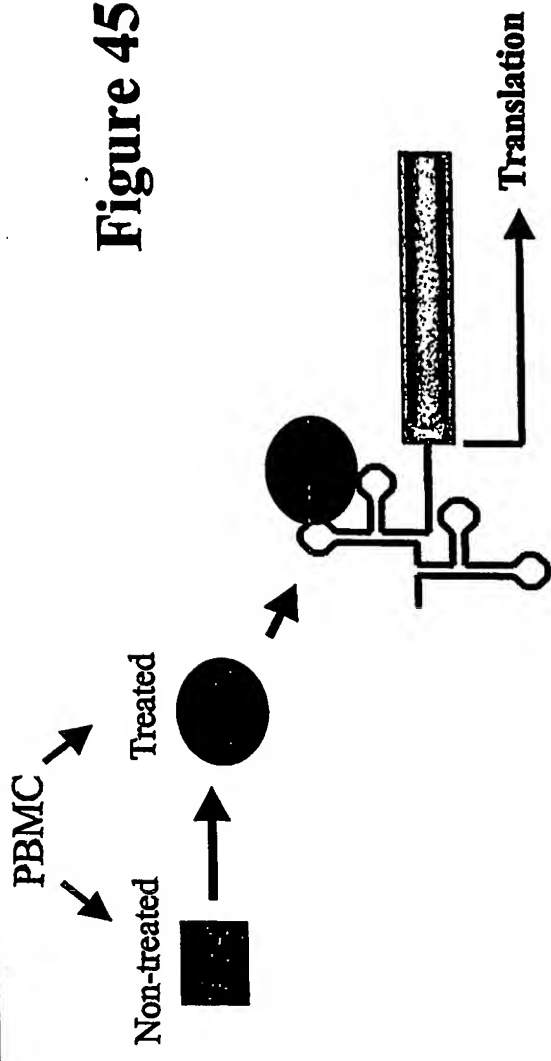


Figure 45

II- Translation inhibitor.

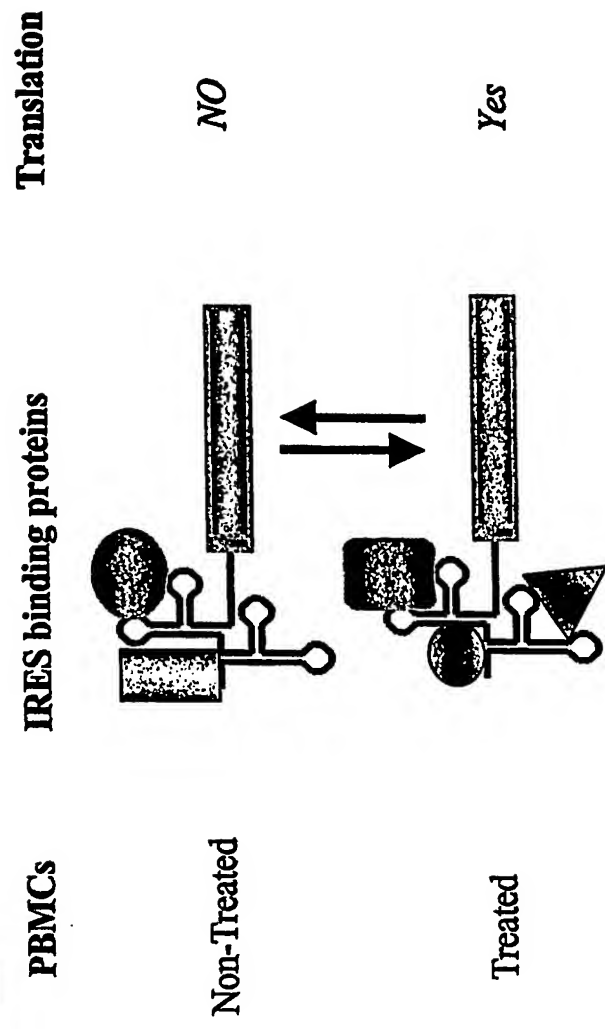


Figure 46

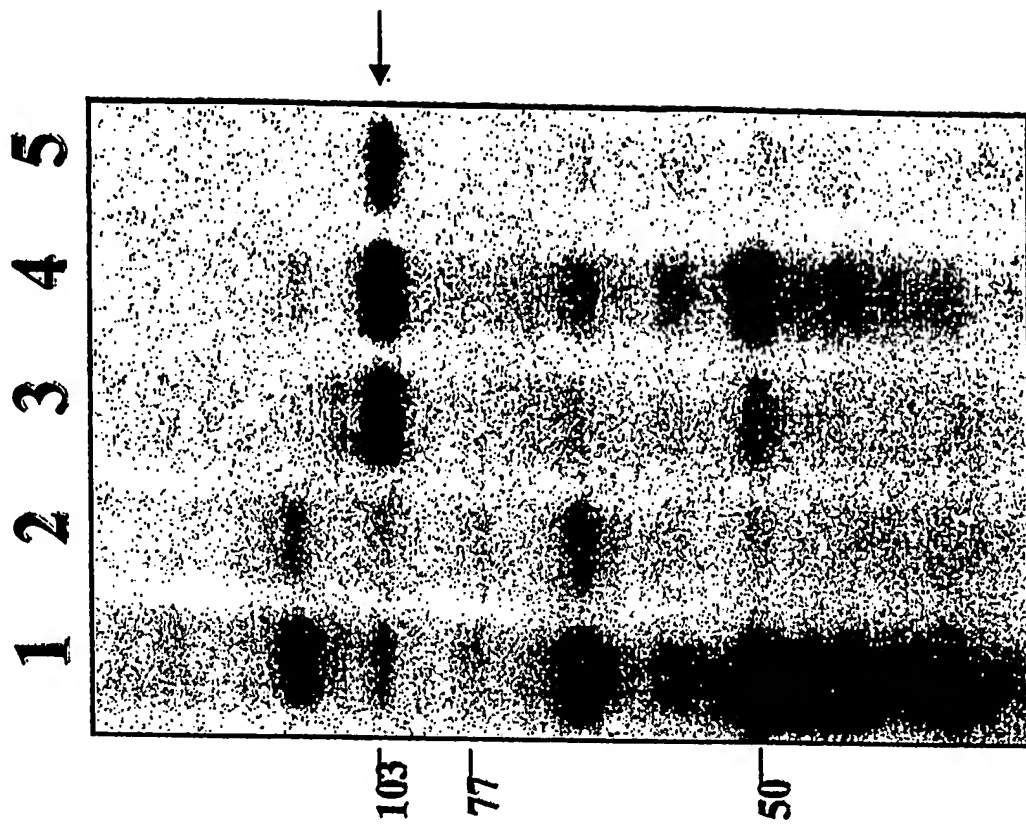


Figure 47

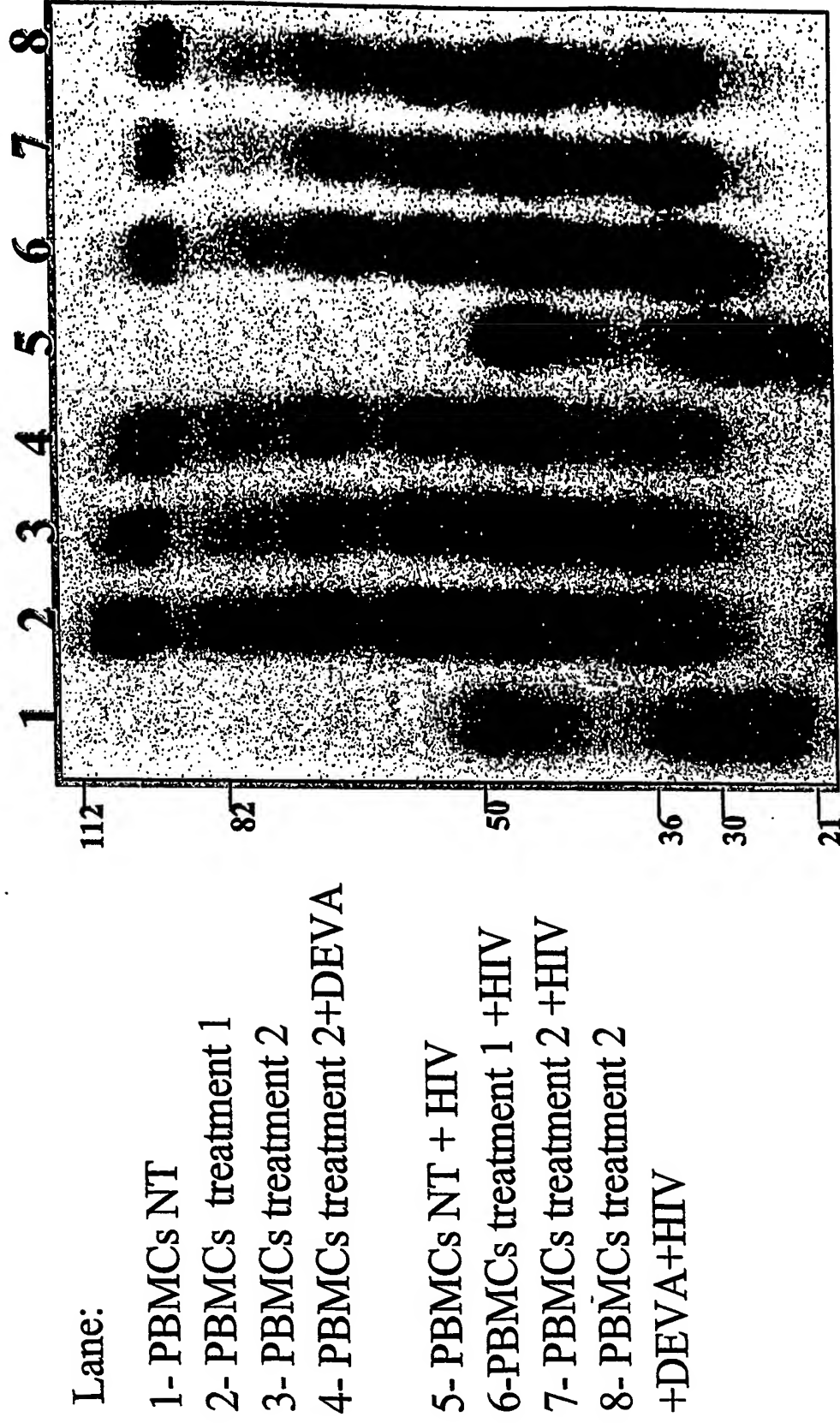
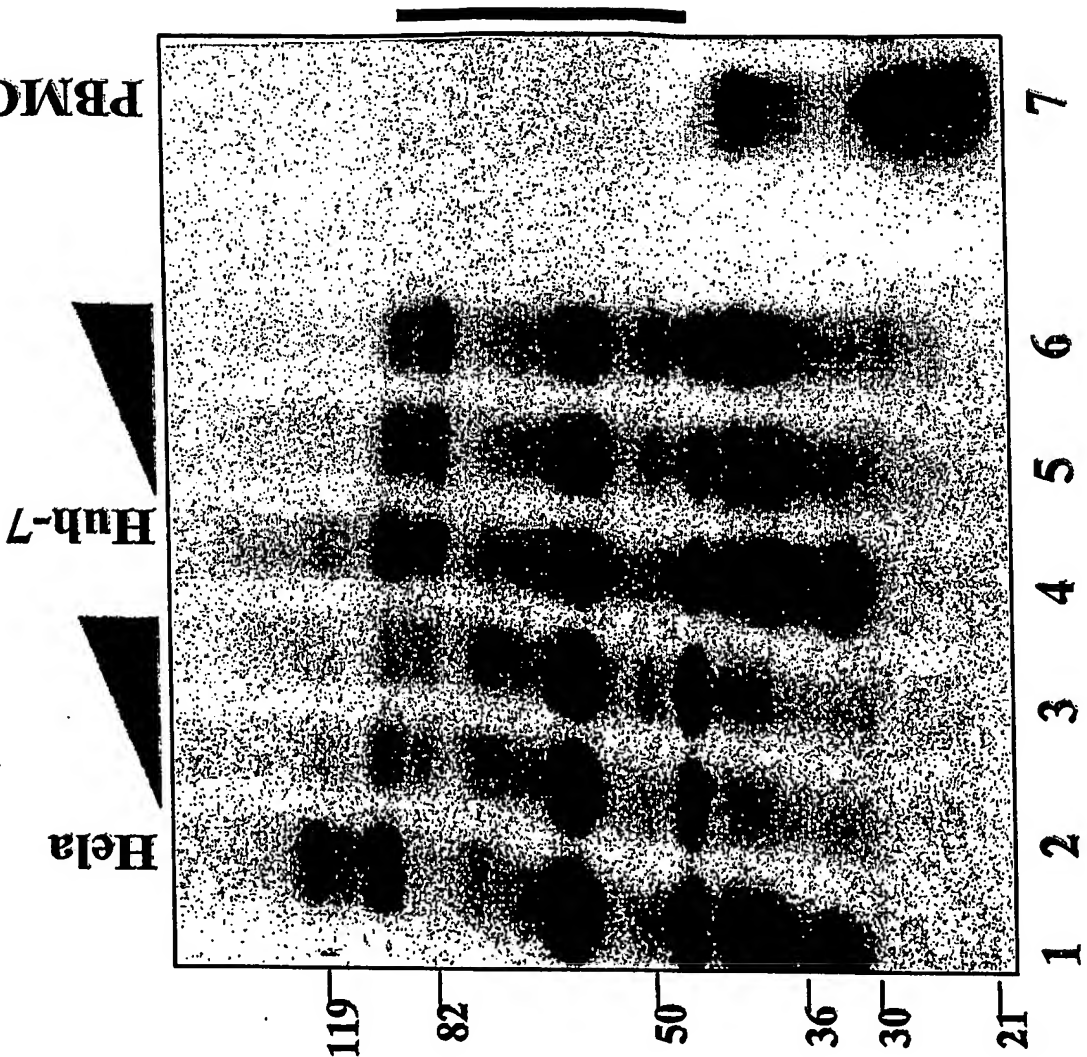


Figure 48



Lane:

- 1- HeLa (40 ug)
- 2- HeLa + PBMCs NT(6 ug)
- 3- HeLa + PBMCs NT (12ug)
- 4- Huh-7 (38 ug)
- 5- Huh-7 + PBMCs NT (6ug)
- 6- Huh-7 + PBMCs NT (12ug)
- 7- PBMCs NT (12 ug)

PBMC are HCV(-)

Figure 49

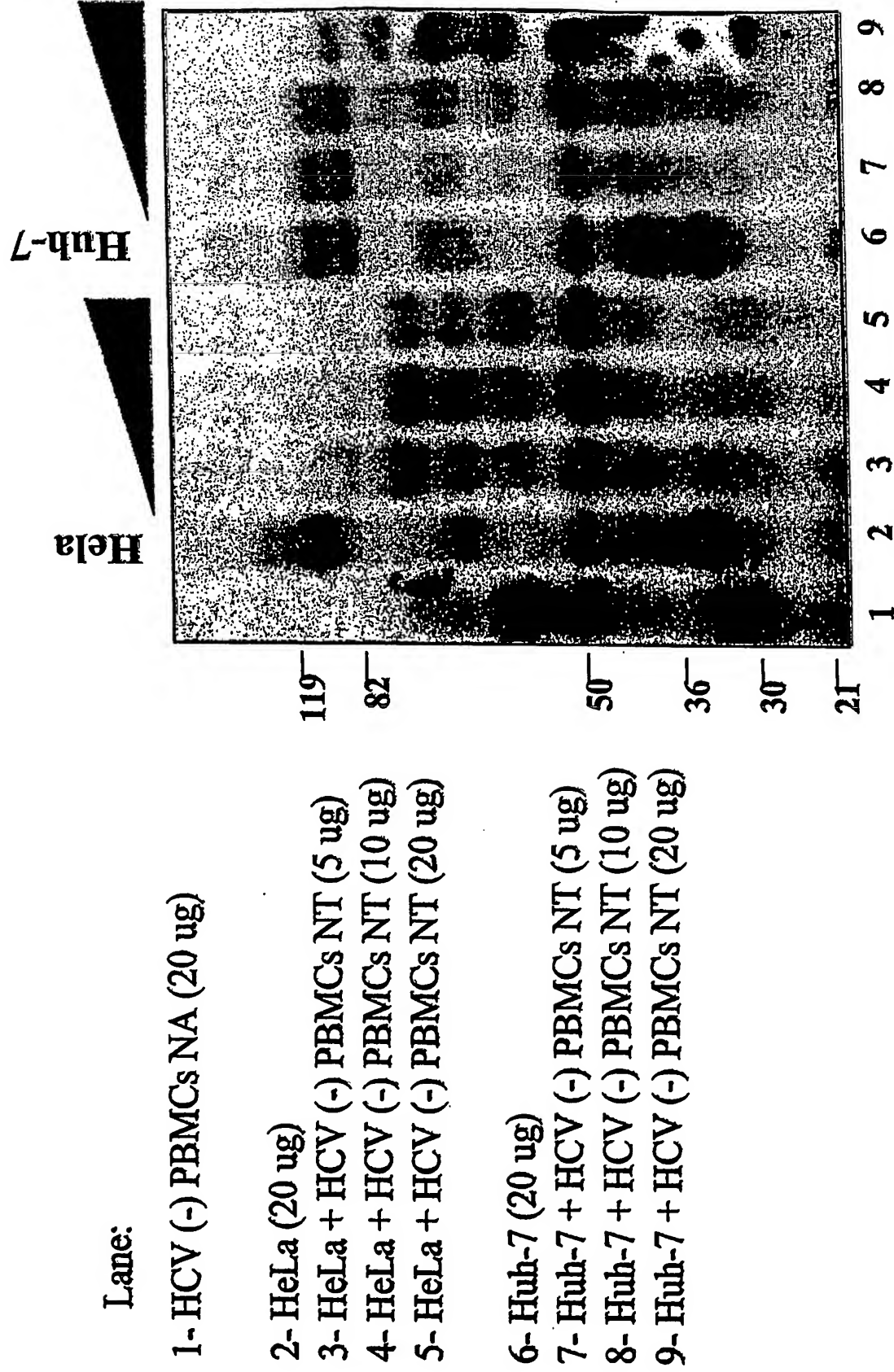
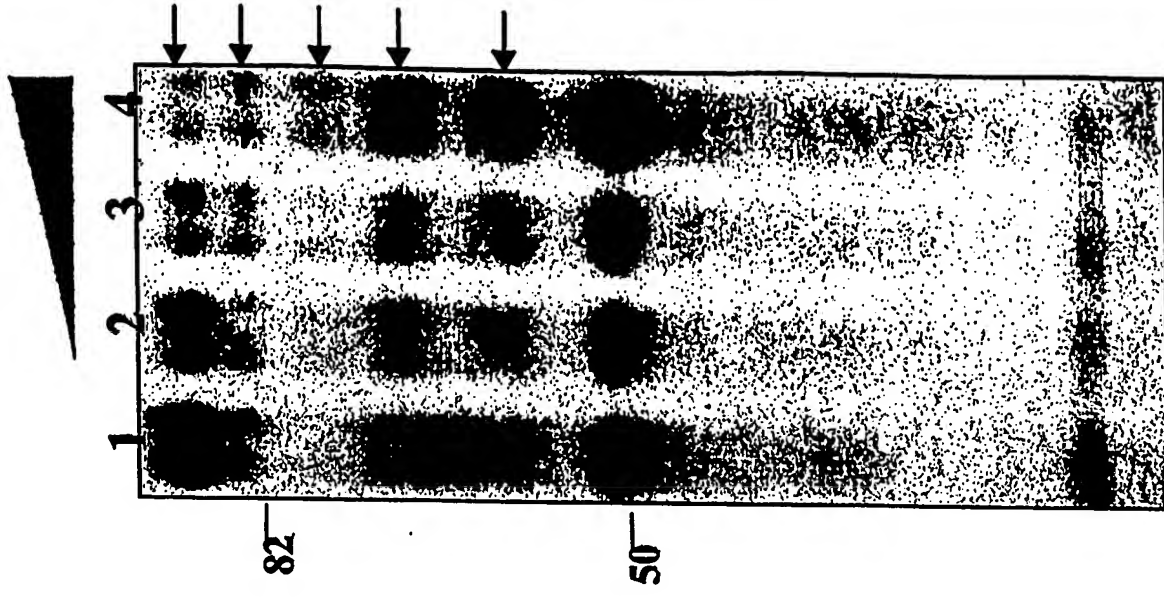


Figure 50

Lane:

- 1- Huh-7 (20ug)
- 2- Huh-7 + HCV (-) PBMCs NT (5ug)
- 3- Huh-7 + HCV (-) PBMCs NT (10ug)
- 4- Huh-7 + HCV (-) PBMCs NT (20ug)



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